CLOTTING ANALYSIS OF BLOOD SAMPLES FROM INTENSIVE CARE UNIT PATIENTS

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ABBREVIATIONS

ρM	picomolar
µM	Micromolar
AA	Arachidonic acid
ADAMST-13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
ADP	Adenosine diphosphate
ANOVA	Analysis of variation
APACHE	Acute Physiology and Chronic Health Evaluation
APCCs	Activated prothrombin complex concetrates
APTT	Activated partial thromboplastin time
AT	Antithrombin
AU	Absorbance units
AUC	Area under the curve
BHK cells	Baby Hamster Kidney cells
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CAT	Calibrated automated thrombography
CFR	Clot formation rate
CFT	Clot formation time
CLoFAL	Clot formation and lysis
Coll	Collagen
CRP	C reactive protein
CT	Clot time
CTI	Corn trypsin inhibitor
DIC	Disseminated intravascular Coagulation
DIT	Drug induced thrombocytopaenia
DMSO	Dimethyl sulfoxide
DMU	DeMont Fort university
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
ETP	Endogenous thrombin potential
FBC	Full blood count
FEIBA	Factor eigt by-passing agent
FEU	Fibrinogen equivalent unit
FEU/L	
FFP	Fresh frozen plasma
FV	Factor V
FVa	Activated factor V
FVIIa	Activated factor VIIa
FVIIIa	Activated factor VIIIa
FX	Factor X
FXa	Activated factor X
FXI	Factor XI

FXIa	Activated factor XI
FXII	Factor XII
FXIIa	Activated factor XII
Gla	gamma-carboxyglutamic
GPIb/IX	Glycoprotein Ib/IX
GPIb-IX-V	Glycoprotein –IX-V
GPIIb/IIIa	Glycoprotein IIb/IIIa
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIT	Heparin induced thrombocytopaenia
HMWK	High molecular weight kininogen
HUS	Haemolytic Uraemic syndrome
ICU	Intensive care unit
IgG	Immunoglobulin G
IIa	Thrombin
IL-1	Interleukin-1
IL-6	Interleukin-6
INR	International normalised ratio
k	The rate of clot formation on the TEG assay
Kk	Kallikrein
LREC	Local Research Ethics Committee
MA	Maximum amplitude
MA	Maximum amplitude
MCF	Maximum clot formation
MCF-t	Maximum clot formation time
MDA	Multi discrete analyser
ML	Maximum lysis
MP	Maximum peak
NaCl	Sodium chloride
ng/ml	Nanogram/mililitre
NR	Normal range
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptors
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PL	Phospholipid
PreKk	Prekallikrein
PS	phosphatidylserine
PT	Prothrombin time
r	Tine taken to clot formation on the TEG assay
rFVIIa	Recombinant activated factor VII
RHuTF	Recombinant human tissue factor
ROTEM	Rotational thromboelastometry
RR	Reference range
SD	Standard deviation
Sec	Second
SEM	Standard error of mean

T1	Time at clot initiation
TAFI	Thrombin activatable fibrinolysis inhibitor
TAT	Thrombin antithrombin
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
tMA	Time to maximum amplitude
TNF-α	Tumour necrosis factor - α
tpa	Tissue plasminogen activator
TTP	thrombotic thrombocytopenic purpura
ttPEAK	Time to the peak
TXA	Thromboxane A
VWF	Von Willebrands factor
WB	Whole blood

ABSTRACT

Introduction: Patients in the intensive care unit have complex haemostatic changes, which may be either procoagulant or anticoagulant. Global assays may reflect a patient's net haemostatic balance and can contribute to pro- or anti-coagulant assessment. In this project, global assays were used to investigate both coagulation and fibrinolysis in samples taken from intensive care unit patients.

Materials and Methods: Comparative clotting analysis was carried out on whole blood and plasma samples from twelve samples from intensive care unit patients. Nine haemophiliac samples and 14 healthy individual samples were used as controls. Several assays were used to assess coagulation in these sample groups. These included coagulation screens, individual factor assays and global assays [calibrated automated thrombography, whole blood and plasma low- dose tissue factor activated rotational thromboelastometry and the clot formation and lysis assay].

Results: Clot initiation in both whole blood and plasma analysis was prolonged in the intensive care unit samples. This was observed in the global assay analysis and was elucidated by the standard laboratory tests such as the clotting screens and the individual factor assays. However, once initiation commenced, the intensive care unit samples showed a clot formation comparable to that seen in healthy volunteers.

Conclusion: Clot formation in intensive care unit patients is abnormal compared to that observed in healthy volunteer samples. Several factors such as the fibrinogen and procoagulant factors influence coagulation and the rate of thrombin production. The clot stability in intensive care unit samples was found to be more robust in comparison to that observed in the healthy volunteer sample group. This study showed that the initiation of coagulation is delayed intensive care unit patient samples but, once started, clot formation was comparable to that in healthy volunteer samples.

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CHAPTER 1

INTRODUCTION

1.1 VASCULATURE: HAEMOSTATIC RESPONSE TO INJURY

Haemostasis, a dynamic process consists of two phases, the primary and secondary phase. Primary haemostasis involves platelet adhesion to a site of vessel wall damage, initiating the formation of a platelet plug. In order for this plug to stabilise, secondary haemostasis is necessary, whereby soluble coagulation factors assemble on the surface of adhered blood cells, leading to the activation of coagulation pathways and the formation of fibrin in accord with local conditions. Most assays are designed to investigate abnormalities in this dynamic system to highlight consequences these abnormalities might cause.

Injuries to vessels cause platelet and coagulation responses that correlate with the degree of injury caused by rectifying the injury without obstructing the vessel. There are multiple mechanisms by which this process occurs. Endothelial cells (ECs) line the lumen of the blood vessels (Figure 1.1) creating an impermeable layer that prevents passive transfer of blood cells, except in the presence of inflammation. Beneath these cells is the sub endothelial layer (tunica media) that contains several components which play a significant role in both primary and secondary haemostasis.

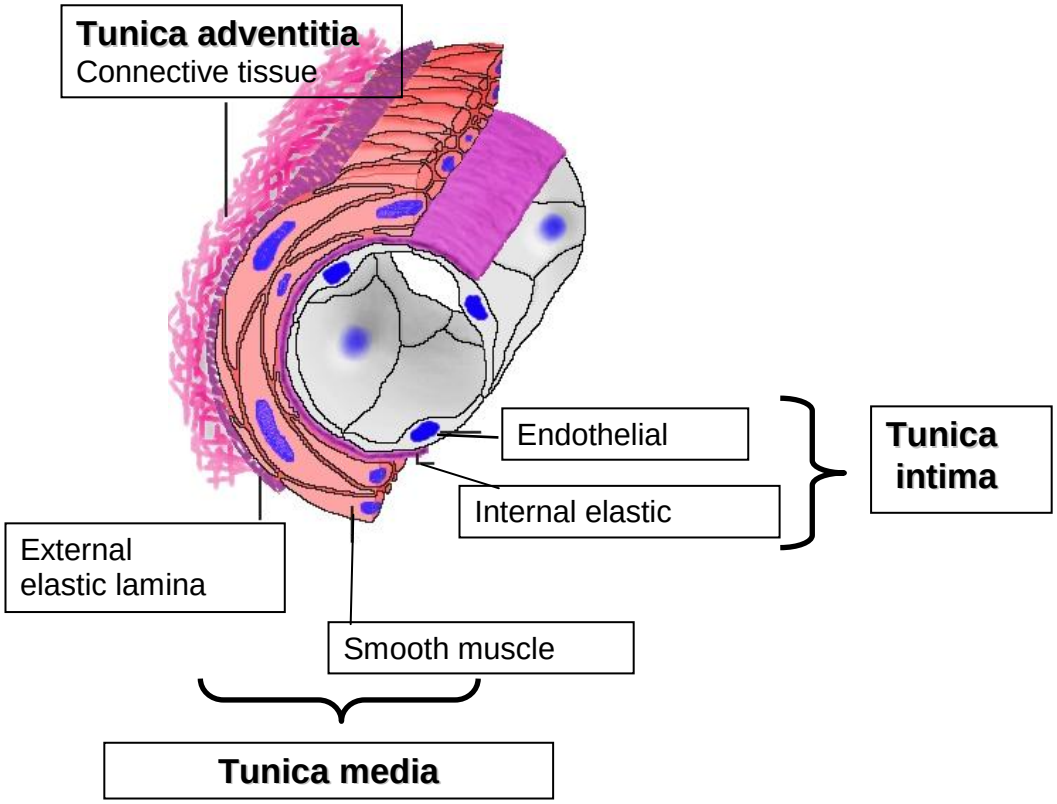


Figure 1.1 Vascular physiology. Diagram adopted from "introduction to histology" (www.siumed.edu)

Unactivated platelets cannot bind to endothelial cells. Any damage that is caused to the endothelial layer exposes the subendothelial matrix which is rapidly covered in stimulated platelets which soon degranulate. In this matrix are components such as collagen IV, V and microfibrils that bind stimulated platelets and result in platelet aggregation. Von Willebrand factor, vitronectin, fibronectin and thrombospondin all have receptor sites that facilitate platelet attachment. All these activities lead to a cascade of events resulting in a system employing both protein and cellular components (Figure 1.1a).

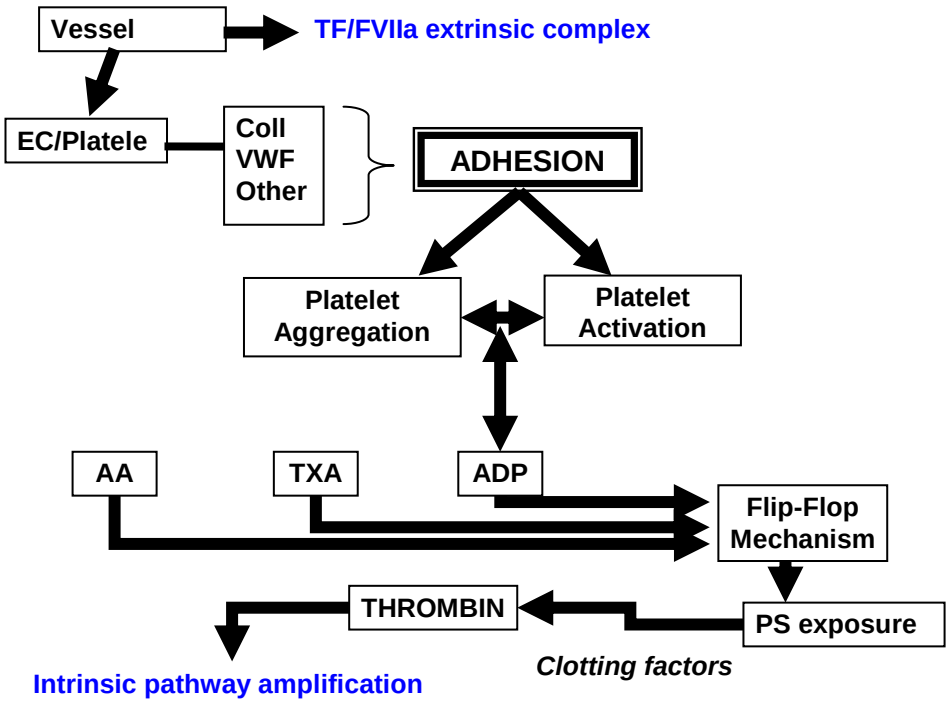


Figure 1.1a Platelet responses to vessel injury. Platelets play a vital role in haemostatic responses to vessel wall injury and are influenced by several factors (e.g extrinsic TF/FVIIa and sub-endothelial components). EC, endothelial cell; TF, tissue factor; Coll, collagen; VWF, von Willebrand factor; ADP, adenosine triphosphate; AA, arachidonic acid; TXA, thromaboxane A; PS, Phosphatidylserine. Figure is based on analogy from Altman *et al*, 2006

1.2 REGULATION OF COAGULATION

Maintenance of normal haemostasis is controlled through surface-expressed molecules on ECs, circulating inhibitors of plasma proteins and negative feedback mechanisms. The main regulator of coagulation is AT (antithrombin) which inhibits thrombin, FXIa (Activated factor XI), FXa (Activated factor X) and FVIIa (activated factor VII). AT is regulated by binding on to heparin sulphate proteoglycans on the EC surface where it forms a highly stable complex that traps the AT from the circulation. Another regulator is TFPI (tissue factor pathway inhibitor) which inhibits the TF/FVIIa/FXa complex.

The second domain of TFPI first binds FXa, and then the first domain binds FVIIa. This prevents further FXa being generated via the extrinsic pathway. Approximately 80% of TFPI is regulated by binding to glycosaminoglycans on EC surfaces. Only a small percentage circulates in plasma and this circulatory TFPI is regulated by binding to low density lipoproteins with its affinity enhanced in the presence of heparin, thus contributing to its regulation.

Thrombin is efficient at physiological restoration of vascular injury. Due to this there is an innate regulatory process that prevents uncontrolled coagulation. Thrombin serves as both a procoagulant and an anticoagulant but this is regulated based on an intact endothelial layer. Thrombin that is produced at the site of an intact endothelial layer binds to thrombomodulin expressed on ECs. Thrombomodulin is a potent modulator of thrombin and a co-factor in the activation of PC (Protein C). PC binds ECs via the endothelial protein C receptor and stimulates its activity by the thrombin-thrombomodulin complex. The resultant activated PC degrades FVa (activated factor V) and FVIIIa (activated factor VIII), reactions that are upregulated by the co-factor protein S (Dahlback, 2000).

Physiologically, both pro- and anti-coagulant mechanisms favour anticoagulation but when vascular damage occurs, anticoagulation is downregulated. Despite this well-controlled system, defects in any part of pathways involved in the generation of thrombin and production of fibrin can cause either thrombotic or haemorrhagic consequences.

1.3 MODELS OF HAEMOSTASIS

1.3.1 Thrombin-based model of haemostasis

In all reported models of haemostasis to date, the common constant is that generation of a stable clot requires a thrombin-mediated conversion of fibrinogen to fibrin. Several *in vitro* models have been designed, simulating *in vivo* processes and have been used to study the development of a defective clot in order to develop therapeutic interventions for bleeding or thrombotic tendencies.

1.3.2 Architecture of a fibrin clot

Currently, mechanisms of fibrin production and clot formation have been studied using models that utilise global assays. These assays differ from clot-based assays, which although they define coagulation defects, they do not discriminate risks in individuals (Brummel-Zeidins *et al*, 2003; Brummel-Zeidins *et al*, 2005). Global assays demonstrate the overall pattern of clot formation. Currently, the two commonly used global assays are the endogenous thrombin potential (ETP) and thromboelastography (TEG). These assays have been evaluated using clot formation profiles within different patient groups and it has been noted that waveform patterns in both ETP and TEG show significant correlation between the velocity of the thrombus tensile strength generation and the velocity of thrombin generation (Rivard *et al*, 2005).

Fibrin formation can be analysed using a turbidimetric assay with the rate of clot formation being recorded as shown in Figure 1.2. The mechanics of the fibrin clot being formed have been recently reviewed (Wolberg, 2007).

Initially, thrombin binds to the fibrinogen molecule, leading to the cleavage of fibrinopeptides attached to the E nodule of fibrinogen. The first strands of fibrin that form (protofibrils) are half-staggered and double stranded but the clot formed at this stage is not stable enough (the lag phase). Thicker fibrin polymers form and aggregate (lateral aggregation), creating an increase in turbidity.

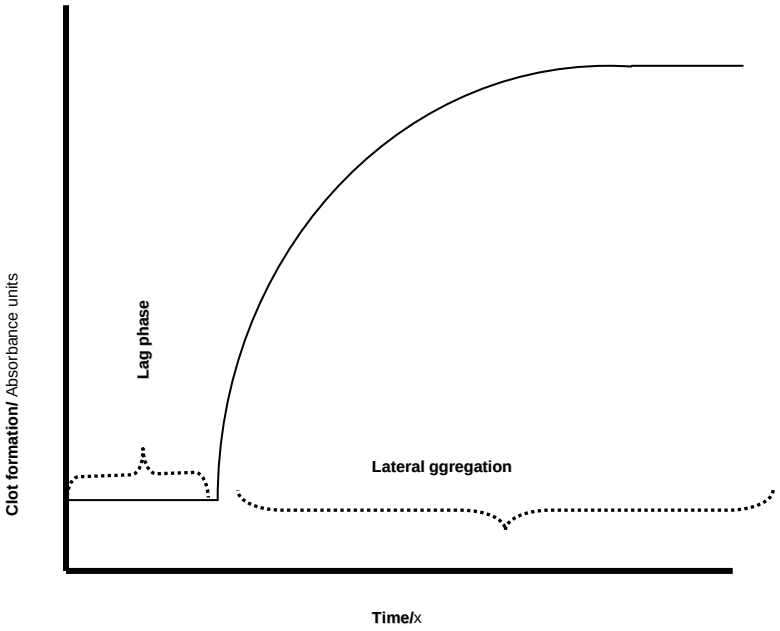


Figure 1.2 Turbidimetric representation of fibrin clot formation. Figure derived from Wolberg, 2007

The concentration of thrombin present influences the structure of the fibrin clot formed. Very low concentrations (<0.1U/ml) are enough to cleave

fibrinopeptides (Wolberg *et al*, 2005; Brummel-Zeidins *et al*, 2005) and allow fibrin polymerisation however they produce clots that are turbid and composed of thick, loosely-woven fibrin strands (Blomback *et al*, 1989). Increased concentrations of thrombin produce thinner, tightly-packed fibres which may indicate that clots with thin fibres are architecturally more stable. The impact this has on the stability of fibrin is most noticeable during fibrinolysis. Fibrin binds plasminogen and tissue plasminogen activator (tpa) to convert the enzyme plasmin that initiates fibrinolysis. Gabriel and his group showed that clots with thicker fibres had an advantageous susceptibility to lysis (Gabriel *et al*, 1992). Thinner fibres have a slower rate of tpa-mediated lysis which means that these clots were more resistant to fibrinolysis. When observed at a fibrin monomer level, it was revealed that thicker fibres lysed slower individually. The increased lysis rate noted during polymer fibrinolysis was attributed to the fact that clots formed in the presence of low thrombin concentrations were made up of fewer fibrin strands despite sufficient levels of fibrinogen and therefore had a high surface area for plasmin binding. This suggests that even though plasmin is formed on monomer fibrin fibres, its activity through the fibrin mesh is rate limited.

1.3.3 Cellular interactions in thrombin generation

Specific cellular surfaces are required in the coagulation process for the purpose of binding zymogens, cofactors and eventually complexes. All these processes occur sequentially in three main stages as described in Section 1.4 according to the new cell-based model of haemostasis.

Initiation of coagulation produces minute concentrations of thrombin, mainly due to FXa. Thrombin levels produced during initiation were found to be in the ranges of 0.5 – 2nM which are sufficient for the rapid activation of platelets, FV, FXIII and fibrin formation, all of which occur before propagation (Brummel *et al*, 2002; Mann *et al*, 2006). It was found that after clotting was started platelet activation followed by FXIII and FV activation was observed at a clot time of 4 minutes and all this activation precedes fibrinopeptides release. After the 4-minute clot time, 25-50% of procoagulant substrates were cleaved and 60% of the platelets were activated. All of this occurred with less than 1% of the total thrombin produced. In conclusion, by the time a clot is visually seen, 25%-60% of reactions involved in the formation of that clot will already have occurred but 96% of the thrombin will yet to be generated. Why thrombin needs to be produced abundantly after this point is as yet not known.

TF is expressed either by damage after endothelial cell damage or cytokine-related exposure, although this is still controversial, and its binding to FVII in plasma initiates coagulation. There are three main ways TF is expressed all which have stimulated a debate on which of these TF sources plays the major role in clot formation. TF has been reported to be expressed on blood cells, contained within circulating microparticles and in a soluble form circulating in blood (Orfeo *et al*, 2005). These three sources raise the question as to whether TF-dependent thrombin generation requires a continuous exposure to TF. This question was answered by Orfeo and his group (Orfeo 2005), first, by explaining that functional TF was essential to trigger thrombin production but

not required for normal thrombin generation once the reactions had been underway for more than 120 seconds. Next, they concluded that there was no active TF in healthy individuals and that a reservoir for TF was unnecessary to sustain coagulation. The clot formed during initiation requires a re-supply of blood, not for more TF, but to immediately renew the consumption of prothrombin. *In vivo*, vasculature is an open system where a resupply of factor and platelets is quite crucial and it is this resupply that governs the extent of the clot formation reaction. Ultimately, the phase of thrombin generation during initiation is brief and because this TF-induced thrombin generation stage happens during an inert stage, it requires re-supply not re-initiation. During amplification, the FXa from the initiation stage produces a small amount of thrombin that activates intrinsic co-factors (FVIII, V) and platelets which exposes phosphatidylserine (PS) and binding sites for other zymogens. Propagation occurs on the surface of activated platelets, employing more intrinsic factors and creating a thrombin burst. This thrombin generates a stable clot and activates FXIII, which along with Tissue factor fibrinolysis inhibitor (TAFI) protects the clot from lysis. The result is a cross-linked network of fibrin polymers with increased elasticity and overall vascoelastic properties. The thrombin-based coagulation process follows a graphical dynamic pattern (Figure 1.3) when considering the concentration of thrombin generated over the course of the process. This graphical representation has been based on *in situ* (Wolberg, 2007) and *in vitro* studies (Al dieri *et al*, 2002; Hemker *et al*, 2004; Dargaud *et al*, 2005; Hemker *et al*, 2006; Wolberg, 2007), whereby a

system was designed to incorporate plasma procoagulants, inhibitors, platelets and TF-bearing cells.

Clot formation can therefore be paralleled to thrombin generation as shown in Figure 1.3. The lag phase (T1) lasts for 2-6 minutes with little thrombin produced on TF-bearing cells but this can still activate FV, FVIII and platelets. The maximum rate (T2) is the propagation phase which lasts for around 10 minutes and the increasing rate corresponds to the "burst" of thrombin production on the activated platelet surfaces. This is followed by the maximum thrombin peak (T3) at which the thrombin-antithrombin (TAT) complexes start to reach equilibrium and the amount of measurable thrombin is stabilised. Subsequently, the production of thrombin ends returning the levels to baseline as a result of TAT formation. The lag phase (C1) represents the initial stage of thrombin generation (initiation and amplification) on the TF-bearing cell, platelet activation (explaining the decrease in turbidity), thrombin production on platelets and protofibril formation. (C2) occurs during the propagation stage when the concentration of thrombin increases (Wolberg, 2007).

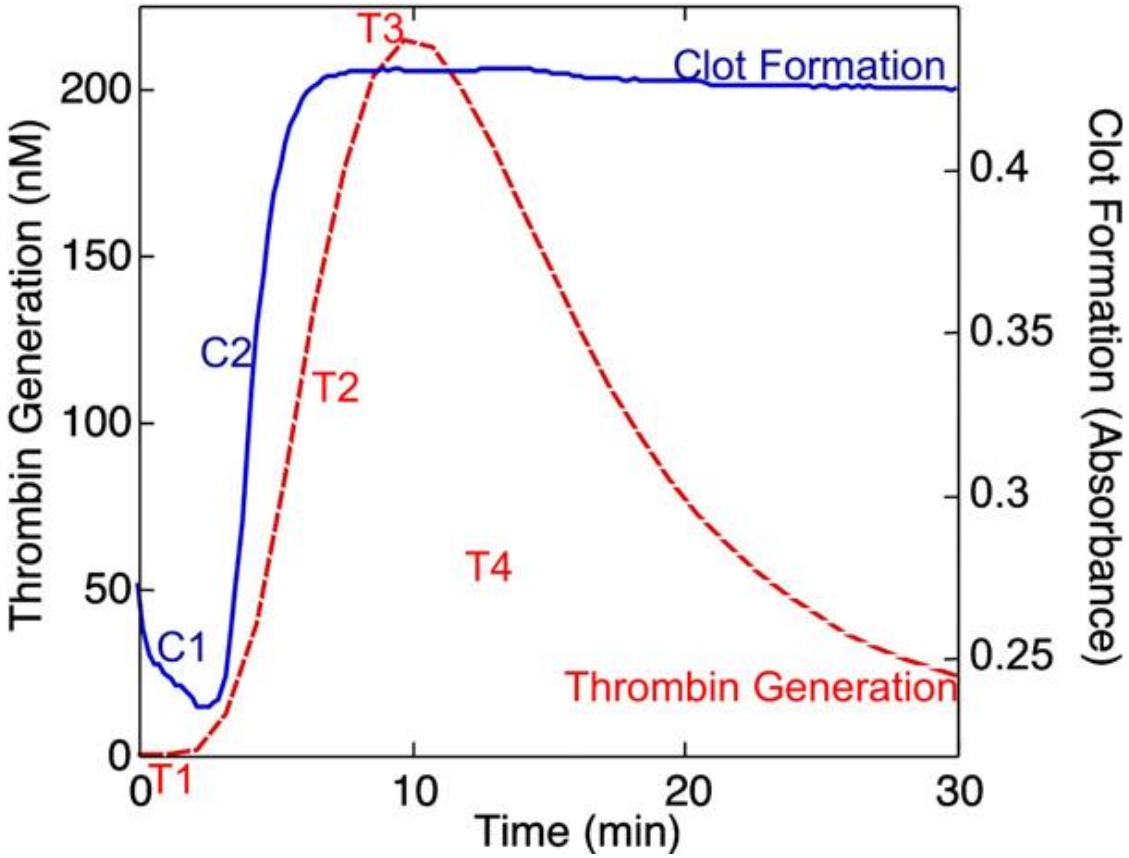


Figure 1.3 Thrombin generation (Blue) and fibrin clot formation (Black). During the generation of thrombin, there are 3 noted phases. The lag phase (T1), maximum rate (T2), maximum thrombin generated (T3) and the total free thrombin produced {area under the curve} (T4). Clot formation can be paralleled to thrombin generation. In clot formation, C1 corresponds to the lag time, lateral aggregation (C2) shows the maximum rate of thrombin regenerated at a steady rate. (Figure acquired from Wolberg, 2007)

Care should be taken when interpreting and comparing data produced from thrombin generation assays (Wolberg, 2007). There are several variables that influence fibrin clot formation especially in some systems where cells are present. Calcium, which is required to assemble procoagulant factors, has been reported to shorten the initiation of the clotting process and produces thicker fibres than when fibrin is produced in its absence. The presence of antithrombin also reduces thrombin levels as it lowers the concentration of free thrombin available (Wolberg, 2007). This will cause prolonged lag times and

ticker fibrin fibres. The presence of other proteins (macro-globular proteins) in plasma will also influence clotting.

1.3.4 Cell surfaces and fibrin structure

The extrinsic and intrinsic pathways play vital roles in the formation of fibrin clots. The extrinsic pathway produces low concentration thrombin on TF-bearing cell surface that triggers the onset of fibrin clot formation (Wolberg *et al*, 2005). Initially, it was believed that this early fibrin mesh was more porous and that later on when more thrombin was produced, a more tightly packed mesh was layered on top of the initial mesh, creating a more condensed and therefore secure clot (Blomback *et al*, 1994). More recently, it has been suggested that after the initial low-thrombin based fibrin clot is formed, the clot itself evolves with the addition of new fibrin strands to the already formed fibrin mesh. These subsequent strands elongate and cross-branch other strands present, creating a dense fibrin clot. This indicates that the final fibrin clot formed is composed of several different sized fibrin fibres.

In conclusion, the presence of procoagulant factors, cellular counterparts as well as thrombin generated during clot formation, all determine the structure and subsequently the stability of the clot.

1.4 CELL-BASED MODEL OF HAEMOSTASIS

Initially, the coagulation cascade was considered to be the classical representation of the coagulation model. It was this cascade model that most

laboratory tests were based on to evaluate haemostatic disorders. This earlier theory of haemostasis originally indicated that coagulation factors controlled haemostasis in a system where cells, particularly platelets, merely provided phosphatidylserine-containing surfaces on which these procoagulant proteins assembled.

This theory however did not explain haemostatic mechanisms *in vivo.* In particular, why certain groups of patients had a bleeding tendency and also it did not predict which patients were at risk of bleeding. An example of this is in patients that are deficient in FXII (factor XII), HMWK (high molecular weight kininogen) or prekallikrein. These patients do not present with a bleeding tendency although they show a prolonged APTT (activated partial thromboplastin time) on analysis. The APTT test reveals dysfunctional factor activity or level, especially in the intrinsic pathway. In patients with FXI (factor XI) deficiency, there is an increased risk of haemorrhage but the APTT is not indicative of the extent to which the patient may bleed even though the bleeding is markedly lower than that seen in haemophilia A or B. The new cellular model of haemostasis incorporates the cellular components of haemostasis and the role they play in achieving haemostasis.

A cell based model has recently been proposed that highlights the role of different cells with similar phosphatidylserine contents and how they are able to play different roles in haemostasis (Hoffman, 2003). The new cell-based model proposes explanations for the varying degrees of haemorrhage encountered in

the presence of factor deficiencies in both the extrinsic and intrinsic pathways. It explains why even though the extrinsic pathway in haemophiliacs is functional there still is pronounced bleeding. In addition, it explains why the extrinsic pathway cannot sustain adequate clot formation to prevent bleeding in haemophiliacs. It acts as a stepping stone to answering some of the questions surrounding various other deficiencies in haemostasis and further highlights that coagulation occurs in three different stages on three different cell surfaces. Initiation occurs on a TF-bearing cell where as in the amplification stage, platelets and co-factors assemble to prepare for the large thrombin burst and the propagation stage occurs on the surface of activated platelets (Hoffman, 2003).

1.4.1 Initiation

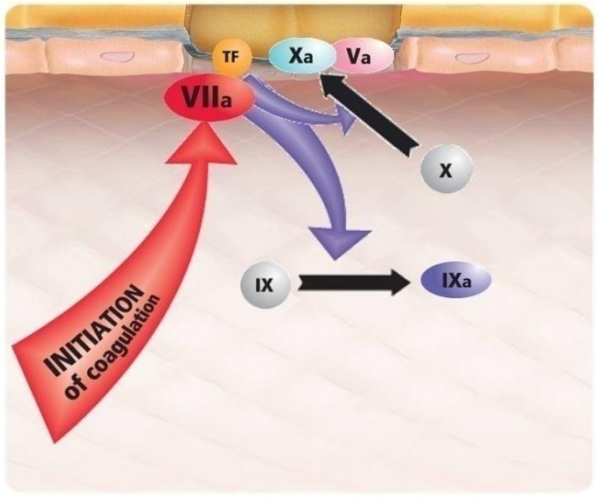


Figure 1.4 Initiation of coagulation (Figure obtained from www.novonordisk.com)

Initiation of coagulation (Figure 1.4) occurs when sub-endothelial tissue is exposed to the circulation at a site of injury. The tissue factor expressed in endothelial cells is released and binds to endogenously activated FVII to form a TF/FVIIa complex. Therefore a cellular source of TF is essential. There has

been evidence (Mann, 1992 and Hoffman, 2003) showing that initiation of coagulation can occur in the vasculature of healthy individuals. This is because certain factors (FVII, FX and FII) infiltrate tissue spaces, depending on their molecular size and are activated by TF. These factors have been detected in lymph and have been assayed along with their activated and inactive peptide forms (Mann, 1992). This may imply that activated factors are present at basal states and that thrombin production can take place outside the vasculature as demonstrated in healthy subjects ((Mann, 1992)) even when the vascular wall in intact. This indicates that the initiation phase is constantly activated contrary to findings of other studies (Orfeo *et al*, 2002), although in order to form a stable clot the link between the vasculature is required. In order to generate a clot, approximately 2nM of thrombin is required. At high concentration of TF, the generation of FXa is dominated by the TF/FVIIa complex rather than the FVIIIa/FIXa complex, as has been shown *in vitro* (Mann *et al*, 2006).

There are several sources of TF, which include stromal fibroblasts, mononuclear cells, macrophages and endothelial cells but unless vessel injury or inflammation occurs, TF does not come in contact with blood. When vessel wall injury is present, the TF/FVIIa complex subsequently produces minute quantities of thrombin.

1.4.2 Amplification

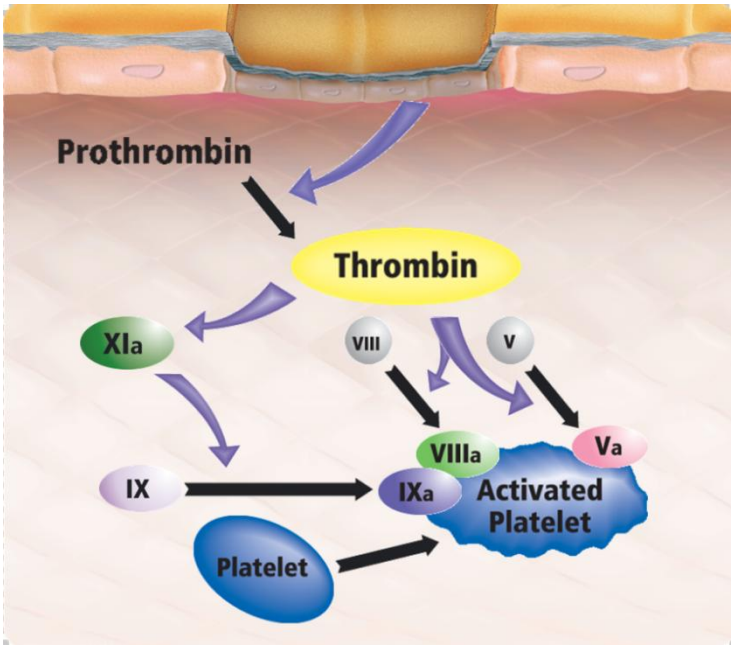


Figure 1.5 Amplification of coagulation (Figure obtained from www.novonordisk.com)

Under normal circumstances, haemostatic components in the vascular tissue cannot escape the endothelial layer because of their size. However, in the presence of vessel wall injury, platelets, FVIII and VWF (von Willebrand factor) leave the vascular tissue and come into close proximity with the small amount of thrombin generated during initiation. Platelets adhere to the injury site and form a plug on the damaged vessel wall. A minute level of thrombin generated (Figure 1.5) activates these platelets by initiating a conformational change through the "flip-flop" mechanism, allowing them to expose their phosphatidylserine-rich surface. This thrombin further activates factor FV released from the activated platelets and cleaves FVIII from VWF.

1.4.2.1 Priming

This component of haemostasis shown in Figure 1.6 is based on a cell model, derived experimentally, from TF sources such as monocytes and fibroblasts as oppose to relipidated TF and also use activated platelets for the generation of thrombin (Kjalke, 1998 and Hoffman, 2001). Priming provides an explanation for the recruitment of platelets and the role they play within the clot.

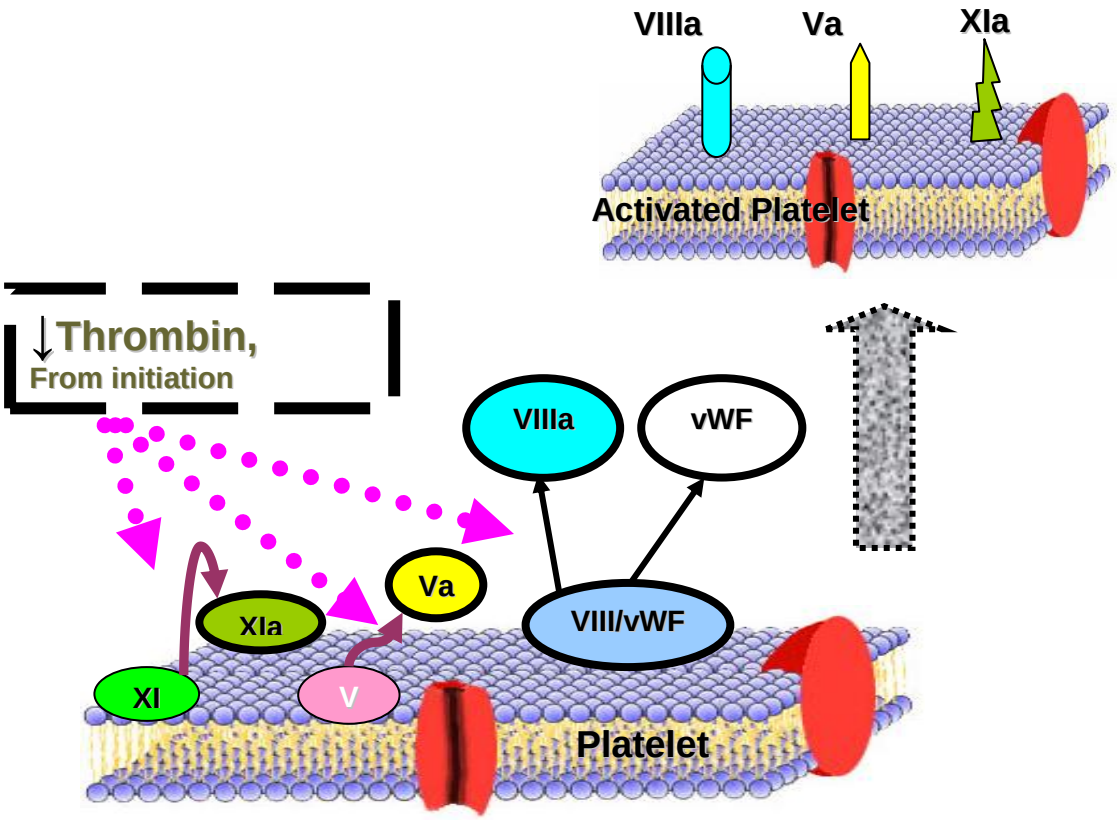


Figure 1.6 Priming in coagulation.
(Figure is derived from Kjalke, 1998 and Hoffman, 2001)

During this stage the TF-generated thrombin produced from the initiation stage binds to the platelets that have adhered to the extravascular matrix tissue, mediated in part by VWF binding to collagen. Binding to matrix proteins like collagen activates the platelets and localises them at the TF site. Thrombin then enhances platelet activation via PAR (proteinase activated receptor)

mechanisms. One of the well-known PAR agonists is thrombin, which is a multifunctional serine protein generated during blood coagulation. Thrombin interacts with cells *via* a specific proteolysis of the extracellular NH_2-terminal of PARs, which leads to exposure of a new tethered ligand, binding intramolecularly to the receptor and initiating signal transduction (Lau *et al*, 1994). Two of the four PARs, PAR1 and PAR4, have been identified in human platelets and are responsible for thrombin-induced platelet activation (Kahn *et al*, 1999). The dual stimulation via collagen and thrombin results in platelet activation higher than that seen with other agonist stimulation. Thrombin-derived stimulation allows platelet degranulation and the subsequent release of FV from platelet α granules. Collagen stimulation of the platelet receptors results in platelet expressing high levels of FV. Thrombin cleaves the partially activated FV to a fully active form. The same cleavage process applies during the activation of FVIII resulting in its release from its co-factor binding molecule von Willebrand factor.

The flip-flop mechanism in platelet activation exposes phosphatidylserine which is required for binding to the γ-carboxyglutamic acid (Gla) residues of the clotting factors in a calcium-dependent manner. TF-generated thrombin binds to specific receptors on platelets to prevent being neutralised by antithrombin. Unactivated platelets have at least 3 binding receptors for thrombin that include the GPIb-IX-V complex that binds thrombin at the heparin-binding site, PAR1 that binds thrombin through the substrate-binding site and anion-binding exocite 1. After the PAR1 is cleaved by thrombin, a new amino terminus with a tethered ligand then binds to other nearby PAR1

receptors, triggering a signalling cascade. PAR4 has shown some signalling capabilities but is mainly linked to platelet aggregation rather than platelet procoagulant activity, which means that its role in haemostasis occurs much earlier during primary haemostasis (Monroe *et al*, 2002). The FVIII-VWF complex allows FVIII to bind to platelets through the VWF binding site on the GPIb-IX-V complex. This brings FVIII in close proximity to thrombin and allows for FVIII activation. FXI binds reversibly to platelets and this binding is enhanced by the presence of prothrombin (Baglia and Walsh, 1998).

Platelet-binding proteins play a major role in propagation. FVa binds tightly to lipids and acts as a binding protein for FXa on the platelet surface. FIXa binds to platelets in the absence of FVIIIa but this depends on the phosphatidylserine content of the lipids (Monroe *et al*, 2003). The result of priming is a platelet that readily binds activated FXIa, cofactors FVa, FVIIIa.

1.4.3 Propagation

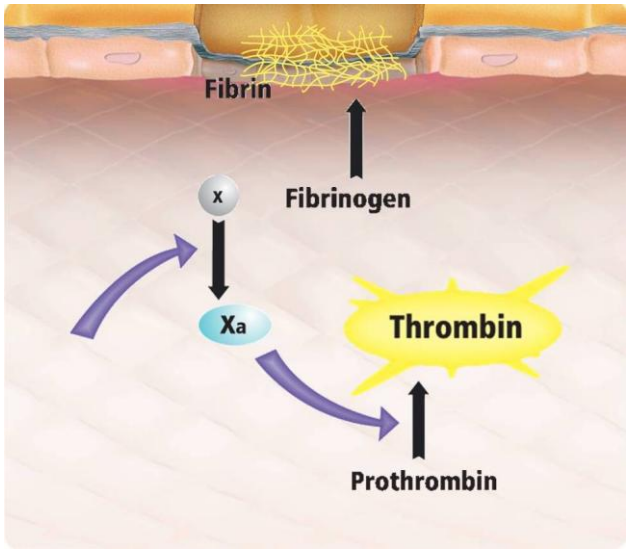


Figure 1.7 Propagation stage of coagulation (Figure obtained from www.novonordisk.com)

At this stage of coagulation, the TF-generated thrombin will have generated several activated proteins that cause a higher concentration of thrombin (approximately 1nM) to be generated during the propagation stage (Mann *et al*, 2006) as shown in Figure 1.7. On the surface of activated platelets, FIXa combines with its co-factor FVIII and this occurs the moment FIXa reaches the platelet surface. The initial FIXa is formed by the TF/FVIIa complex and can move to the platelet surface because FIXa is not rapidly inhibited by TFPI, and AT only inhibits it at a very slow rate (Hoffman, 2003; Monroe *et al*, 2006). FIXa can also be produced on platelet surfaces by FXIa. Tenase complex activates FX to FXa which immediately combines with FV, forming a more protective complex away from TFPI and AT, even in the presence of heparin. This Prothrombinase complex converts large amount of prothrombin to thrombin, which cleaves f fibrinogen to fibrin monomers that polymerise and fuse the platelet plug into a stable clot. The cell based-based model allows a more thorough understanding of how the coagulation system works and provides a larger degree of consistency with clinical observations when it comes to coagulation disorders.

1.5 COAGULATION ABNORMALITIES IN CRITICALLY ILL PATIENTS

There are several predisposing factors and conditions that compromise the haemostatic status in critically ill patients, which subsequently may lead to multiple organ failure in the absence of therapeutic intervention. Many of these patients develop

abnormalities in haemostasis of variable degrees, from minor ones such as prolonged clotting times and isolated thrombocytopaenia to complex ones such as disseminated intravascular coagulation (DIC). There are several underlying causes that may be responsible for disturbed coagulation in critically ill patients and they each may require specific therapeutic intervention. Parameters, like prolonged coagulation times, reduced levels of coagulation inhibitors, or increased fibrin degradation products, all indicate alterations in coagulation that can be easily measured and managed specifically.

A large proportion, 35-44%, of critically ill patients suffers thrombocytopaenia with a platelet count of under $150x10^9$/L (Vanderschueren *et al*, 2000; Baughman *et al*, 1993; Strauss *et al* 2002). This incidence is higher in surgical or trauma patients where approximately 41% of these patients have less than $100x10^9$/L platelets (Stephan *et al*, 1999 and Hanes *et al*, 1999). The main clinical significance of thrombocytopaenia is that it is related to an increased risk of bleeding. In addition to this, a stable reduction in platelet counts is an indication of active coagulation, which in turns contributes to microvascular failure and organ dysfunction. However, regardless of the cause, thrombocytopaenia in critically ill patients is an independent predictor of ICU mortality (Vanderschueren *et al*, 2000 and Strauss *et al*, 2002) in that patients that maintain a sustained drop in platelets show a 4- to 6-fold increase in mortality. The severity of thrombocytopaenia in critically ill patients is inversely proportional to the survival rate and studies have shown that the platelet count is a stronger indicator of ICU mortality than the standard composite scoring systems like the Acute Physiology

and Chronic Evaluation (APACHE) II score (Vanderschueren *et al*, 2000 and Acka et al, 2002).

Prolonged clotting times (like the prothrombin time (PT) and activated partial thromboplastin time (APTT)) occur in approximately 28% of critically ill patients (Chakraverty *et al*, 1996 and Macleod *et al*, 2003). Trauma patients have a high incidence of prolonged times and studies have shown that the presence of a prolonged PT and/or APTT is a strong independent predictor of mortality (MacLeod et al, 2003). Other indicative tests include elevated fibrin degradation products and reduced levels of inhibitors such as protein C and antithrombin.

1.5.1 Thrombocytopaenia

Thrombocytopaenia in critically ill patients is caused by different factors. The most common factors include sepsis, DIC, massive blood loss, thrombotic microangiopathy and heparin- or immune- or drug-induced thrombocytopaenia.

a) Sepsis

Sepsis is the host's response to infection and this is characterised by the release of inflammatory mediators that culminate into a wide range of responses (such as hypothermia, increased oxygen consumption, tachycardia, hyperglycaemia and lactic acidosis as well as liver and renal function alteration). In septic patients, the main factors that contribute to thrombocytopaenia are impaired platelet production, increased consumption or destruction, or platelet sequestration in the spleen. Even though there is a high circulating level of thrombopoietin, there is an impaired production of platelets from the bone marrow. In critically ill patients

haemophagocytosis may occur where megakaryocytes and other haemopoetic cells are cleared from the bone marrow by monocytes and macrophages in response to the macrophage colony stimulating factor (Francois *et al*, 1997). Thrombin is the main activator of platelets *in vivo* and thrombin production is a ubiquitous event in sepsis even with or without the presence of DIC.

b) Disseminated Intravascular Coagulation

Patients with DIC have a low or decreasing platelet count (Levi and ten Cate, 1999). However the presence of DIC complicates other underlying conditions such as sepsis, trauma or cancer and the impact of DIC in critically ill patients is further discussed in section 1.5.3

c) Thrombotic microangiopathy

Thrombotic microangiopathy defines a group of syndromes (HUS [haemolytic-uremic syndrome], TTP [thrombotic thrombocytopenic purpura], severe malignant hypertension and chemotherapy-induced microangiopathy), which all have a common pathogenic denominator that is endothelial damage. Endothelial damage causes platelet adhesion and subsequently aggregation. The result of this endothelial damage-induced platelet aggregation is thrombocytopaenia, mechanical fragmentation of red cells as they are forced through the clot mesh at high pressure and obstruction of the microvasculature of organs such as the brain and kidneys, which leads to renal failure and neurological dysfunction. All of these syndromes have different aetiologies. In TTP, it is the absence of von Willebrand cleaving enzyme ADAMTS-13, that leads to ultra-large multimers attached to the endothelial cells binding to platelet receptors and causing platelet adhesion and aggregation. In HUS, a cytotoxin released from gram-negative bacteria causes endothelial cell and platelet activation. In severe malignant

hypertension and chemotherapy-induced microangiopathy, it is the direct mechanical stress of the high pressure and the chemical stress that cause the endothelial damage.

d) Heparin- and drug-induced thrombocytopaenia (HIT and DIT)

Administration of heparin carries a 5% risk for the development of HIT in patients receiving heparin, although this is dependent on dosage and duration of administration. HIT is caused by an antibody binding to heparin platelet factor-4 complex expressed on the surface of the platelet. This results in high elevation of platelet activation and therefore consumptive thrombocytopaenia. A small proportion, 1%, of critically ill patients given heparin will develop HIT (Verma *et al*, 2003) but this is also dependent on whether they are given low molecular weight heparin or unfractionated heparin, the later having a higher risk of HIT development.

DIT may be caused due to drug-induced myelosuppression or as a result of immune-mediated reactions to the drugs. DIT is more difficult to diagnose as critically ill patients are usually exposed to a myriad of drugs and other causes for thrombocytopaenia. Usually, the diagnosis is based on eliminating other causes, calculating when the thrombocytopaenia developed and which drug was introduced at the time.

1.5.2 Prolonged global clotting times

PT and APTT are global assays but do not thoroughly reflect the *in vivo* haemostatic picture but they conveniently give an estimation of coagulation factors for which either the PT or the APTT is sensitive. Generally, if the factors levels are below the lowest reference value for these tests, then the times will be prolonged, but it should be noted that the sensitivity of these tests varies markedly depending on the reagents

used. As a result an international normalised ratio (INR) is used by most centres to allow greater standardisation between laboratory centres. This parameter has only been validated for control of the intensity of anti vitamin K dependent therapy (Kitchen and Preston, 1999).

a) Impaired synthesis

In most critically ill patients, factor deficiency is usually due to consumptive coagulopathy or impaired synthesis or development of a factor inhibitor as seen in acquired haemophilia. Impaired synthesis is usually due to liver insufficiency or vitamin K deficiency. The PT is sensitive to both conditions and especially sensitive to FVII. FV is not a vitamin K-dependent factor whereas FVII is. This means that FVII deficiency is more indicative of liver failure than FV. Measurement of both these factor levels can be used to identfy the presence of liver impairment.

b) Consumptive coagulopathy

Massive blood loss due to trauma or surgery is one of the ways that uncompensated factor loss may occur. This is also the case where massive blood loss has been compensated by volume replacement using crystalloids, colloids and red cells units without factor replacement. Trauma patients tend to develop hypothermia, which underestimates coagulation *in vivo* since most of the laboratory tests are standardised at 37°C. Factor consumption also occurs in a DIC setting.

1.5.3 Disseminated Intravascular Coagulation

There are three critical events within DIC. They include thrombin production, the mechanism sustaining the production of thrombin and the associated activation of the inflammatory cascade.

a) Inflammatory link to coagulation

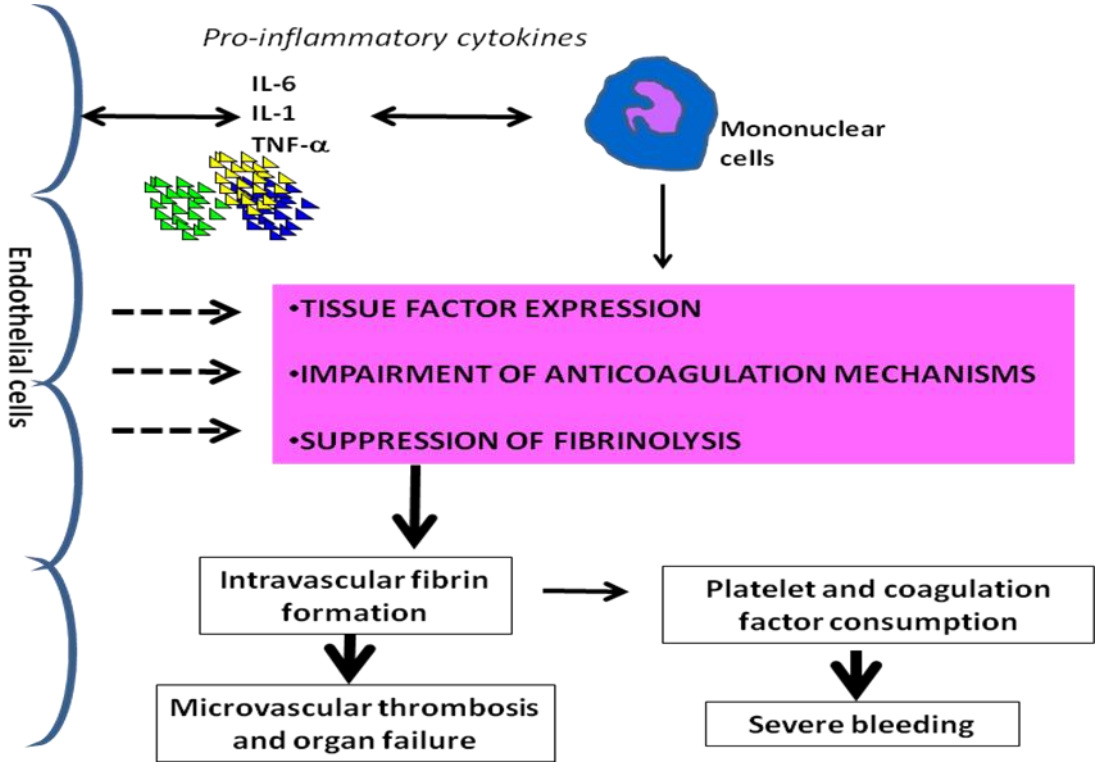


Figure 1.8 Activation of coagulation after inflammatory response. Figure derived from Levi, 2002

DIC occurs frequently as a complication of severe sepsis, trauma and several other conditions. DIC is a condition that is associated with patients in the ICU setting and is a result of several haemostatic abnormalities in these patients. These abnormalities are not rare in the ICU setting with a higher incidence in the medical than in the surgical setting. Rate of mortality relates to the platelet count but mortality is dependent on bleeding where platelet counts are involved. Bacterial infections are also common in patients that need intensive care and so are responsible for the most common combination that causes coagulation abnormalities, namely sepsis and DIC. Combined sepsis and DIC play a significant role in subsequent depletion of platelets and subsequently abnormal coagulation.

Procoagulant and inflammatory response to infection are closely linked. Infectious agents induce the release of inflammatory cytokines such as IL-1, IL-6 and TNF-α that activate coagulation by simulating TF release from monocytes and damaged endothelium. TF leads to the formation of thrombin and a fibrin clot (Figure 1.8). Inflammatory cytokines and thrombin both impair the endogenous fibrinolytic system by releasing PAI-1 from platelets and endothelium. PAI-1 inhibits tissue plasminogen activator (tPA), which endogenously lyses fibrin. This means that the lytic process is downregulated. In addition to this, thrombin itself stimulates TAFI activation which prevents clot lysis. This is vital in microvascular thrombosis.

The inflammatory response also affects the activation of protein C. Protein C requires thrombin-bound thrombomodulin and since the damaged endothelium will result in low levels of expressed thrombomodulin, the end result is usually the development of diffuse endovascular injury, microvascular thrombosis, organ ischaemia, multiorgan dysfunction and possibly death.

Studies have shown that critically ill patients have a microbiologically confirmed infection and these bacterial infections were found to be the most common cause of hospital acquired infections, with staphylococci being the most common blood isolates and *Candida Albicans* being the most common pathogen isolated from Urine (Foreman *et al*, 2003 and Osmon *et al*, 2003). In lung injury, there is increased permeability of the alveolar-capillary membranes, alveolar damage and accumulation of pulmonary oedema that contains a high concentration of proteases. There is also extensive epithelial necrosis, swollen endothelial cells with widened intracellular

junctions, with fibrin deposits in the hyaline membranes of the alveolar ducts and air spaces. Later stages of lung injury show massive neutrophil and macrophage infiltration as well as fibrinous thrombi in the alveolar capillaries and smaller pulmonary arteries (Toh and Dennis, 2006). These studies have helped identify the overall impact of infections on patient outcomes, especially hospital mortality

b) *In vivo* thrombin generation and its preservation

Thrombin generated *in vivo* is controlled by the protein C pathway and several other mechanisms that control surface expression and signalling on endothelial cells. Although tissue factor and the intrinsic pathway influence thrombin generation, the burst of thrombin generated from the intrinsic pathway also results in the consumption and depletion of protein C, antithrombin and protein S. The increased exposure of cellular surfaces rich in phosphatidylserine provide externalisation of the inner cell surface membrane and induce apoptosis. Cell damage releases microparticles from platelets, monocytes and endothelial cells, all of which increase the availability of phosphatidylserine-exposed surfaces on which coagulation reactions occur. Low density lipoprotein is present in sepsis and acts as a negatively charged surface on to which coagulation occurs (Toh *et al*, 2002).

Critically ill patients with DIC have a low and decreasing platelet count, prolonged clotting screen, low levels of plasma coagulation factors and/or increased fibrin degradation products (*Levi et al*, 2002). Therefore, a collection of tests is considered when diagnosing DIC i.e. Platelet counts, clotting screen, D-dimer assay and fibrinogen level. These four tests are designed as the DIC score and are used as a

strong and independent predictor of mortality in critically ill patients with DIC (Dhainut *et al*, 2004).

1.5.4 Other coagulation defects in critically ill patients

The tests mentioned above in regards to diagnosing conditions in critically ill patients sometimes may not reveal any significant defects, in particular platelet dysfunction or even hyper-fibrinolysis.

Critically ill patients with renal dysfunction or severe liver failure frequently have platelet dysfunction and so do patients that are taking anti-platelet drugs such as aspirin or patients that are taking non-steroid inflammatory drugs or potent thrombin inhibitors (e.g. hirudin). To date, there is no accurate routine test for platelet function in critically ill patients and assay methods such as the platelet function assay, which equates to the bleeding time, is imprecise (Forestier et al, 2002).

In patients that have specific types of cancer (e.g. acute promyelocytic leukaemia) hyper-fibrinolysis is frequently present, although generally it is not that common in critically ill patients. Patients that are treated with thrombolytic agents will have induced hyper-fibrinolysis. Increased hyper-fibrinolysis is detected through raised levels of fibrin degradation products and low fibrinogen levels. Other indicators include low levels of plasminogen and α2-antiplamsin.

1.6 RECOMBINANT ACTIVATED FACTOR VII (rFVIIa)

1.6.1 Development and Production of rFVIIa

Recombinant human coagulation Factor VIIa (rFVIIa) improves haemostasis by activating the extrinsic pathway of the coagulation cascade. Like endogenous FVIIa, rFVIIa is a vitamin K-dependent glycoprotein consisting of 406 amino acid residues (MW 50 KDa) that is structurally similar to human plasma-derived FVIIa. NovoSeven is supplied as a sterile, white lyophilized powder of rFVIIa in single-use vials. It is a delipidated protein that requires a phospholipids surface such as that provided by activated platelets and damaged endothelial cells.

The liver is the primary site of FVII synthesis in humans. The production, therefore, of recombinant human FVIIa requires a mammalian expression system so that a protein with the necessary post-translational modifications, such as γ-carboxylation and glycosylation, can be produced. Using a mammalian cell system derived from hamster kidney cells, rFVIIa is produced in large amounts. It is produced as a single chain that is spontaneously activated during processing, at the purification stage (Jurlander *et al*, 2001).

The amino acid sequence and posttranslational modifications of rFVIIa from the cell line were compared to human plasma FVIIa and found to be identical, with regard to the primary structure, for the amino acids sequence and identical to that predicted from the cDNA sequence. In rFVIIa, nine of the ten Gla residues are fully γ-carboxylated and one partially γ-carboxylated and the two

O-glycosylated sites are similar but the O-linked structures differed slightly between the plasma-derived FVIIa and the recombinant form. Quantitatively, the carbohydrate composition is different in that rFVIIa has a higher fucose content and lower sialic acid content than plasma-derived FVIIa (Thim *et al*, 1988). The low sialic acid content of the recombinant protein may explain the properties of rFVIIa since sialic acid is mainly found on glycolipids where its presence is responsible for the negative charge on the surface of animal cells. However, the low sialic acid content in rFVIIa explains the delipidated properties of the protein and why it requires a negatively charged surface to bind TF and FX.

1.6.2 Introduction of rFVIIa use in coagulopathies

Severe haemophiliacs require prophylactic therapy with a haemostatically effective treatment to avoid chronic anthropathy development as a result of regular joint bleeds. This treatment is also used if patients suffer trauma or surgical-related bleeds. Approximately, 20% of severe haemophiliacs develop an antibody against the factor that they lack and prophylactic replacement of the deficient factor may not be as effective as the factor is neutralised by the existing antibody. Much research has focussed on finding therapies that target mechanisms that bypass the FVIII/FIX factors, which deficient in haemophilia A and B patients. APCCs (Activated Prothrombin Complex Concentrates) contain both activated factors and their zymogens and are still widely used as FVIII bypassing agents, but they only give 50-65% effective responses in the patients (Sjamsoedin *et al*, 1981; Lusher *et al*, 1983) with some

thromboemobolic events reported (Lusher, 1991). After Hedner and his co-workers looked at canine models to identify the factors most involved in the development of these side effects, they concluded that FVII/FVIIa was a useful haemostatic candidate that utilises a pathway independent of FVIII/FIX (Hedner *et al*, 1979). Further work by this group demonstrated that purified plasma-derived FVIIa induced haemostasis in severe haemophiliacs through therapeutic physiological levels of FVIIa binding TF and activating FX at the site of injury (Hedner and Kisiel, 1983). rFVIIa has since been used to enhance coagulation in haemophiliacs with inhibitors and is now being used in other coagulopathy conditions besides haemophilia.

1.6.3 Pre-clinical development

The haemophilic canine models that were used to test the hypothesis that rFVIIa can be effectively used in circulation showed an appropriate physiological response to these rFVIIa dosage without any systemic activation of haemostasis after the administration (Brinkhous *et al*, 1989). This was based on several hypothetical factors. Firstly, no specific inhibitors to FVII have been identified and so it should not be cleared so readily from circulation. Secondly, in the absence of TF, the rFVIIa will be inactive in circulation. The third point is that at the injury site, TF is generated as a cell-membrane receptor for FVIIa and the TF/FVIIa complex forms promptly to initiate the extrinsic pathway, which indicates that TF may be essential for rFVIIa to work. Lastly, the extrinsic pathway in haemophilia is normal. Consequently, the administration of rFVIIa in haemophilia could allow for the generation of

sufficient thrombin and platelet activation to facilitate the formation of a stable plug. A comparative study that was conducted in rabbits showed that FEIBA (Factor Eight Inhibitor By-passing Activity) significantly lowered the platelet counts and fibrinogen while increasing the APTT, demonstrating an overall consumption of coagulation factors (Diness *et al*, 1992). This was not the effect found with rFVIIa.

VWD homozygous type I or type III canine models did not show any improvement in haemostasis even at higher doses indicating the importance of a platelet adhesion and aggregation for the full action of rFVIIa (Brinkhous *et al*, 1989; Roberts *et al*, 2004). The defect in these models was severe and was primarily attributed to defective platelet adhesion and agglutination resulting from the absence of VWF.

1.6.4 Clinical development

It was during an open surgical synovectomy in 1988 that the first severe haemophiliac was treated with rFVIIa without any complications or peri- or postoperative abnormal bleeding. After this, an efficacy rate of 90-100% was confirmed in relation to several severe haemophiliac patients undergoing major surgery (Ingerslev *et al*, 1996 and Shapiro *et al*, 1998). Other reviews have reported efficacy rates in limb- and life-threatening bleeds to be between 76%-84% in severe haemophliacs with inhibitors (Lusher *et al*, 1998; Abshire and Kenet, 2004 (a); Lusher *et al*, 1998 and Key *et al*, 1998). rFVIIa was approved for use in haemophilia with inhibitors in 1996 (Europe), 1999 (USA) and 2000 (Japan).

1.7 TREATMENT AREAS: CONGENITAL AND ACQUIRED HAEMOPHILIA

1.7.1 Haemostasis in haemophiliac patients

Congenital haemophilia A and B is inherited in an X-linked recessive manner and is defined by the reduction or the absence of FVIII, FIX respectively (Haemophilia C is inherited in an autosomal recessive manner and is characterised by a reduction or absence of FXI). The lack of these factors results in inefficient activation of FX on the platelet surface and so there is no amplification stage of coagulation (Figure 1.5). Over the larger part of the 20th century, haemophiliacs were managed with replacement therapy of the deficient factor from products derived from donor human blood products. Some of these patients developed antibodies to the replacement factors, which is why the development of rFVIIa has proved vital in these patients.

Acquired haemophilia is a spontaneous autoimmune disorder where patients with previously normal haemostasis develop IgG auto-antibodies directed against clotting factors. Most antibodies develop against FVIII, but FIX auto-antibodies are less common. Development of antibodies against other clotting factors is even rarer. Part of the treatment includes immunosuppressants and treatment with factor replacement, rFVIIa and activated prothrombin complex concentrate (e.g FEIBA).

1.7.2 Rationale behind bleeding in haemophilia

The cell-based model gives a better understanding of the pathophysiological responses observed in haemophiliacs. With the classic coagulation cascade

model (i.e. the non cell-based model), the indication is that the TF pathway does not produce sufficient FX to sustain haemostasis since in haemophiliacs there is less or no FXI and FVIII to activate. The FX generated at the Tenase complex is not sufficient for the thrombin burst observed during amplification. The reason for the insufficient FX production is accounted for when the cell-based model is applied.

The explanation given by the cell-based model (based on Hoffman, 2003) isn't that the TF-generated FXa is insufficient but rather that it occurs on the wrong cell surface. The FIXa/FVIIIa complex activates FX on the platelet surface during propagation and the TF/FVIIa activates FX on the TF-bearing cell. FXa produced on the TF-bearing cell cannot move to the activated platelets because it is liable to inhibition by two major plasma FXa inhibitors (AT and TFPI). During circulation, both TFPI and AT inhibit FXa so rapidly and effectively that the half-life of this protein is 1 minute or less in the fluid phase. FXa that remains at the TF-bearing cell is protected from inhibition, where as any FXa that diffuses from this cell surface is quickly inhibited.

This would suggest that haemophilia is a result of failure of platelet-surface activation of FX, i.e. a lack of platelet-surface thrombin production. The initiation and amplification stages of coagulation are relatively normal which means that they can form the initial platelet plug but cannot generate a thrombin burst at the platelet surface that is necessary to stabilise this initial plug.

1.7.3 Use of rFVIIa in haemophiliac patients

Replacement therapeutic responses have proven useful in the management of congenital haemophilia. However, alloantibodies to these products in some patients created somewhat of a challenge initially, before the development of FVIII/FIX bypassing agents. Recombinant FVIIa has a structural homology to endogenous plasma-derived FVIIa. rFVIIa use was first investigated experimentally using plasma-derived FVIIa as treatment in haemophiliacs with inhibitors (Hedner *et al*, 1989) but now rFVIIa has been indicated for the treatment of patients with acquired haemophilia A and B with an efficacy of 90-95% (Von Depka, 2002, Von Depka 2005). The efficacy of this drug is not determined by the inhibitor levels and unlike plasma-derived products, it does not cause a renewed rapid antibody production on the second (or subsequent) encounter. Several case studies and reports have shown that rFVIIa use is useful in individuals without any pre-existing coagulopathies; as those seen in patients with acquired haemophilia (Hedner, 2002). Recombinant FVIIa induces haemostasis independent of FVIII or FIX in patients with acquired haemophilia as studies have shown (Lusher *et al*, 1998; Hedner and Ingerslev, 1999). The standard recommended dose is 90-120μg kg^{-1} given every two hour with increasing dose intervals, depending on the clinical outcome of the patient (Hedner and Ingerslev, 1998; Lusher *et al*, 1998; Roberts *et al*, 2004; Von Depka, 2005). Most plasma proteins have a short half-life and the same applies to rFVIIa. The half-life differs depending on whether the patient is a child (3-15 yrs) or an adult (18-55 yrs) as indicated in a pharmacokinetics study (Villar *et al*, 2004) and a case report (Cooper *et al*, 2001). The $T_{1/2}$ was

reported at an average value of 1.32hours (1.27-1.40hours) in children and 2.72 hours in adults (Cooper *et al*, 2001). The high clearance rates noted in patients indicates that a higher does of rFVIIa is required to generate the amount of thrombin required to compensate for the lack of FVIII/FIX in haemophilia.

Inhibitor development has been reported to occur in early childhood (9-36 exposure days) which is why it is vital to investigate inhibitor development in children (Villar *et al*, 2004). There is literature on the response of adult haemophiliac patients to rFVIIa but not to the same extent in children. There have only been two groups that have investigated the pharmacokinetics of rFVIIa in children (Hedner *et al*, 1998 and Santagostino *et al*, 2001) and both have shown a faster clearance of the drug in children than adults. This high level of clearance was attributed to extracellular and total body water spaces being relatively larger in children than in adults (Villar *et al*, 2004).

Monroe *et al*, 1997 and Khalke *et al*, 1998 showed that rFVIIa concentrations of 5nmol L^{-1} or higher bound to surfaces of activated platelets and that a concentration range of 50-150nmol L^{-1} produced thrombin comparable to that seen in controls and at a similar rate. This is essential for a tight fibrin plug to be formed and so the dose of rFVIIa given to these patients is crucial. An improved clinical response with only one single infusion has been reported and may require a higher dose in some patients (Abshire and Kenet, 2004 (a)), as doses as high as 320μg kg^{-1} have been administered to achieve an improved clinical outcome.

Recent reviews within surgical literature on the use of rFVIIa for life-threatening bleeding have shown that there are thromboembolic complications associated with rFVIIa administration (Warren et al, 2007 (a); Warren et al, 2008 (b) and Despotis et al, 2008). A recent paper reported from a review, of various medical disciplines, carried out by the Food and Drug administration that 1 in 50 patients administered with rFVIIa for an unlicensed indication developed a thromboembolic complication and that 1 in 200 patients died (Gill et al, 2009). In cardiac surgery, of the patients that were unresponsive to standard transfusion products and were administered with rFVIIa, 19-40% suffered mortality and complication as a result of using rFVIIa (Gill et al, 2009). These reviews have shown that despite the effectiveness of this drug, there are risks associated with it. This further highlights the need for well-organised randomised controlled trials to set up effective guidelines.

1.7.4 High-dose rFVIIa use in haemophilia

The first report of a "mega dose" of rFVIIa was first reported in 1994 in a boy with haemophilia B and high-titre inhibitors (Cooper *et al*, 2001). Several studies have been conducted to investigate high-dose rFVIIa, especially with regard to thrombotic implications. These studies have shown that "mega dosing" in rFVIIa use provides a large degree of haemostatic efficacy in patients that lack response to standard doses and that it also allows a dosing flexibility that is specific to the patient and related to the likelihood of bleeding (Abshire and Kennet, 2004 (a); Kenet *et al*, 2003). Although mega doses have proven successful in paediatric patients, it has been recommended that the

standard dose of 90μg/kg every 2-3 hours should be maintained in adult patients until safety data has been collected (Abshire, 2004 (b)). Further support for high dosing has been reported in cases where doses as large as 352μg/kg (haemophilia B) and 246 and 986μg/kg (on five consecutive days for haemophiliac A patient) did not show any subsequent increased thrombotic potential (NovoSeven prescribing information) although recent literature has shown that there is a thromboembolic risk associated with rFVIIa (Warren et al, 2007(a) and Despotis et al, 2008).

1.7.5 Mode of action of standard and high-dose rFVIIa

If patients do not have a pre-existing coagulopathy then rFVIIa will not have any effect on the circulatory levels of platelets or fibrinogen but it will increase fragment 1 + 2 and Thrombin-Antithrombin (TAT) complexes (Friederich *et al*, 2003). This is attributed to the diffusion of rFVIIa into the extravascular spaces at the site of vessel wall damage where it binds TF and increases F1 and F2 levels. This allows TF to initiate coagulation at the site and enhance thrombin production. Furthermore, rFVIIa is localised at the injury site and through TF-independent binding to activated platelets that adhere through von Willebrand factor. If high levels of rFVIIa are present, then factors X and IX can be activated on the surface of these activated platelets (WIlbourn *et al*, 2003). Hoffman, 2003 also explains that rFVIIa *in vivo* undergoes weak association with activated platelets especially at high doses; it is not TF-dependent but rather platelet dependent. The TF-dependent theory explains why rFVIIa is localised at the injury site and for these reasons it may not have

any thrombotic implications but it does not explain the need for high doses. by The weak association of rFVIIa in the TF-independent mechanism is improved by high doses of rFVIIa. If TF-independent binding is of a weak affinity, then high level doses are required to produce effective haemostasis. It should be mentioned that this TF-independent mechanism does not substitute the TF-dependent mechanism. TF is still required to initiate coagulation.

1.8 OTHER TREATMENT AREAS

1.8.1 FVII deficiency

Historically, FVII deficiency was treated with APCCs or plasma-derived FVII concentrates. A study was conducted where 17 FVII deficient patients were given rFVIIa after spontaneous bleeding episodes, major and minor surgical procedures. Haemostasis was secured in the surgical procedures as well as the bleeding episodes and the only side effect was the development of an inhibitor against rFVIIa in one patient (Mariani *et al*, 1999; Scharrer, 1999). This study showed that rFVIIa has efficacy in FVII-deficient patients. It is also safe since any risk of viral transmission is eliminated in its production.

1.8.2 Congenital thrombocytopaenias

Congenital platelet dysfunction disorders are a group of rare disorders that include two that are characterised by the lack of glycoprotein complexes (GPIIb/IIIa in Glanzmann thrombasthenia and GPIb/IX in Bernard-Soulier syndrome). These individuals have a severe mucocutaneous bleeding diathesis and have historically required platelet transfusions to correct their haemostasis. There are several complications related to this treatment such as transfusion reactions and antibody development to missing glycoproteins as well as transfusion-related infections from the blood products.

The first case report of rFVIIa use in a severe epistaxis in a boy was first reported in 1996 (Poon *et al*, 1999) and since then additional cases where rFVIIa has been used in bleeding and surgical prophylaxis in patients with

platelet disorders have been reported (Gerotziafas *et al*, 2004; Almeida *et al*, 2003; He *et al*, 2005; Galan *et al*, 2003; Goodnough, 2004). The exact mechanism of how rFVIIa works in platelet dysfunction disorders remiains unclear but the current proposal is that rFVIIa increased thrombin generation on the platelet surfaces by direct activation of FIX and FX and a positive feedback mechanism activates the platelets while compensating for the qualitative and quantitative defects (WIlbourn *et al*, 2003; Almeida *et al*, 2003).

Several report have shown that rFVIIa at high doses activates FX and FIX on the surface of platelets by a TF-independent mechanism (Monroe *et al*, 1998 and Hoffman *et al*, 1998) which explains how rFVIIa normalises haemostasis in haemophilia. Butenas *et al*, 2002 published data indicating that TF was absolutely essential for coagulation activation by rFVIIa but it was later clarified that these conflicting data differed as a result of different methodologies (Hoffman *et al*, 2002) although both views carry great implications for the use of rFVIIa, especially its dosing.

1.9 GLOBAL ASSAYS IN HAEMOSTASIS

In order to diagnose a coagulation disorder, an understanding of the clinical manifestations as well as a thorough haemostatic investigation is required to come to an accurate diagnosis. Various standard assays within the laboratory give a specific biochemical diagnosis but this does not mean that the outcome correlates with the clinical phenotype of the individual. An ideal laboratory test would be one that can determine the clinical implication of the biochemical diagnosis. This test should also be useful in monitoring the procoagulant as well as the anticoagulant effects of therapy administered. However, there is no current laboratory test that can reflect the overall *in vivo* haemostatic profile of an individual because the development of such an assay is not feasible. However, laboratories have employed assays with the potential to highlight and correlate both clinical phenotype and clinical efficacy of most haemostatic treatments.

1.9.1 Clotting screens

Currently, several analysers have been developed that rely on the principle of using photometric signalling to assess clot formation. Using the multi-disciplinary analyser as an example, results from this device are represented graphically, showing an optical profile of light transmittance waveform as a function of time defined as a clot waveform. APTT (Activated partial thromboplastin time) waveform analysis has proven beneficial in defining the severity of haemophilia when compared to the one-stage factor assay or the clotting APTT assay (Shima *et al*, 2002). The usefulness of this assay has also

been reported in monitoring haemostatic effects in haemophiliac patients given rFVIIa (Shima, 2004).

1.9.2 Thromboelastography/Thromboelastometry

Thromboelastometry is based on Thromboelastography. It is an alternative version of the classical assay developed by Hartet in 1948. Both the thromboelastograph and thromboelastogram are used to measure elasticity of blood by continual graphic representation of the firmness of a blood or fibrin clot during its formation and subsequently fibrinolysis. Since 1996, the term TEG has been used to describe the assay performed using the Haemoscope device (IL, USA) and alternatively, the device marketed by Pentapharm (Basel, Switzeland) uses the term thromboelastometry for the measurement process and ROTEM for the device and traces (Luddington, 2005). The descriptive parameters for both devices carry similarities and are shown in Table 1.1.

PARAMETER	ROTEM	TEG	Definition	Unit
CLOT POLYMERISATION PARAMETERS				
Measurement period	RT	-		
Clot time (time to 2mm clot amplitude)	CT	R	Time from test analysis to 2mm MA	**s**
Period from 2-20mm clot amplitude	CFT	K	Period within which the MA reaches 20mm, represents the dynamics of the clot	**s**
α angle	α (angle of tangent at 2mm clot amplitude)	α (slope between r and k)	Angle between the baseline and the a tangent to the clot curve at 2mm MA	**Degree (°)**
Max. Angle	CFR	-	Angle between the baseline and the tangent at max. slope	**Degree (°)**
Maximum clot strength	MCF	MA	MA reached during test	**mm**
Time to max. clot strength	MCF-t	TMA	Time from CT to MCF	**s**
Clot amplitude (at set time points)	A (5, 10…….)	A (30, 60…….)	Clot firmness at a respective point after CT	**mm**
Clot elasticity	MCE	G (Shear elastic modulus strength) G is a calculated parameter. It increases exponentially as compared to the amplitude. It gives a more sensitive resolution at high amplitudes	Parameter derived from MCF. Dispersal of MCE is better as a parameter at high MA than MCF	
CLOT LYSIS PARAMETERS				
Max. clot lysis	ML	-	Max. lysis detected during run time, difference between the MCF and the lowest amp. After MCF (% of MCF)	**%**
Clot lysis (at set time points)	LY (X) (30, 45……..)	CLI (X) (30, 60……..)	Lysis index at time x. Ratio of amplitude and MCF at given time X after CT	**%**
Lysis time	CLT (10% from MCF)	TTL (2mm drop from MA)	Time from CT until clot firmness is decreased to 10% of the MCF during fibrinolysis (Amp. At time x/MCF*100)	**s**
CLR	CLR (Max. tangent post-MCF)	-	strongest lysis, angle between the baseline and the tangent to the declining firmness curve at the minimum of the 1st derivative	**Degree (°)**

Table 1.1 Terminology used for TEG and ROTEM parameters Information derived from Luddington, 2005 and ROTEM manual, Pentapharm GmbH, 2002/2005

This assay provides information about the initiation and propagation kinetics, fibrin/platelet interactions, the stability of the clot in regards to how firm the clot is and its susceptibility to lysis. This has been well established in fields such as hepatobiliary and cardiac surgery, obstetrics, and trauma medicine and also to identify bleeding risk post-operatively (Kang *et al*, 1985; Tuman *et al*, 1987; Kang *et al*, 1987; Speiss *et al*, 1995; Kaufmann *et al*, 1997). Currently, the main use of the TEG is to monitor blood component therapy during surgery (Luddington *et al*, 2005). This highlights the development this device has undergone since when it was first used as bedside monitor for whole blood.

The TEG offers the possibility of bedside testing, giving a result within 30 minutes regarding the function of platelets, plasma procoagulant and anticoagulant ability and the nature of clot lysis. This gives an indication of which blood components need to be administered. Hence, if the clotting time is prolonged, this may indicate fresh frozen plasma as a therapeutic option. The strength of the clot is largely influenced by platelet function and fibrinolysis may indicate the need for anti-fibrinolytic intervention e.g. tranexamic acid use, if accelerated. Recently, an audit showed that the TEG demonstrates a much faster availability of results and a reduced requirement of blood products when compared with coagulation laboratory assays, especially when looking at peri-operative bleeding (Anderson *et al*, 2003). The main setback with this assay as a diagnostic tool in laboratories is the lack of agreement with standard laboratory variables (Zuckerman *et al*, 1981). In addition, citrated blood is required within a laboratory setting. Comparatively,

native whole blood and citrated blood parameters differ and this has been attributed to incomplete inhibition of activation of the coagulation cascade (Zambruni *et al*, 2004). It is therefore vital to interpret TEG parameter results with a knowledge of sample conditions.

Within the laboratory setting, this assay has been used to assess clinical conditions that other conventional coagulation assays could not adequately test for, such as hypercoagulability and thrombosis risk (O'Donnell *et al*, 2004) as well as haemophilia and its response to rFVIIa and APCCs (Hayashi *et al*, 2004; Sorensen and ingerslev, 2004)

1.9.2.1 Measurement principle

The TEG or ROTEM define various parameters that portray the dynamics and size of the clot during clot formation and lysis (Figure 1.8). The sample is incubated at 37°C in a heated cup and inside the cup, a suspended pin connected to a detector system is immersed in the sample. This is a torsion wire in the TEG and an optical detector in the ROTEM. The pin and cup oscillate alternately to each other through an angle of 4.75°. The movement is instigated from either the cup in the TEG system or the pin in the ROTEM system. As the clot forms, the transmitted rotation between the cup and pin (TEG) or the impedance of the rotation of the pin (ROTEM) is detected and a trace (Figure 1.9) generated.

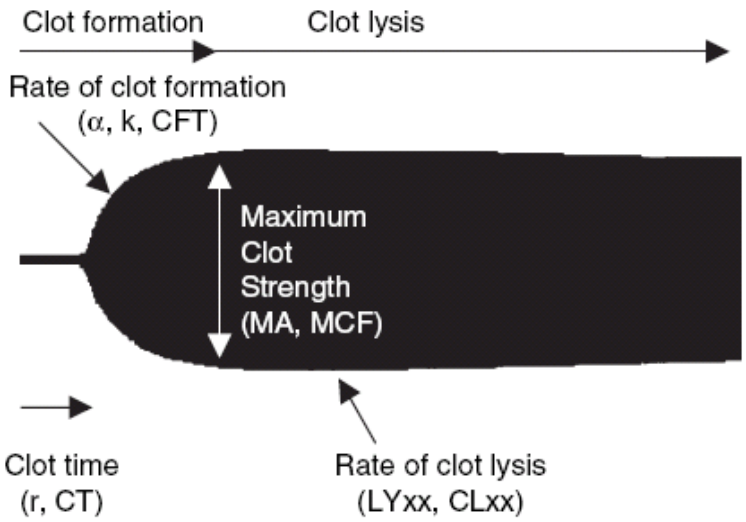


Figure 1.9 Main parameters of the TEG (r, k, and MA) or ROTEM (CT, CFT, and MCF). Figure adapted from Luddington, 2005

Thromboelastography/thromboelastometry has demonstrated that in certain pathological conditions, distinctive traces are generated. These are shown in Figure 1.10

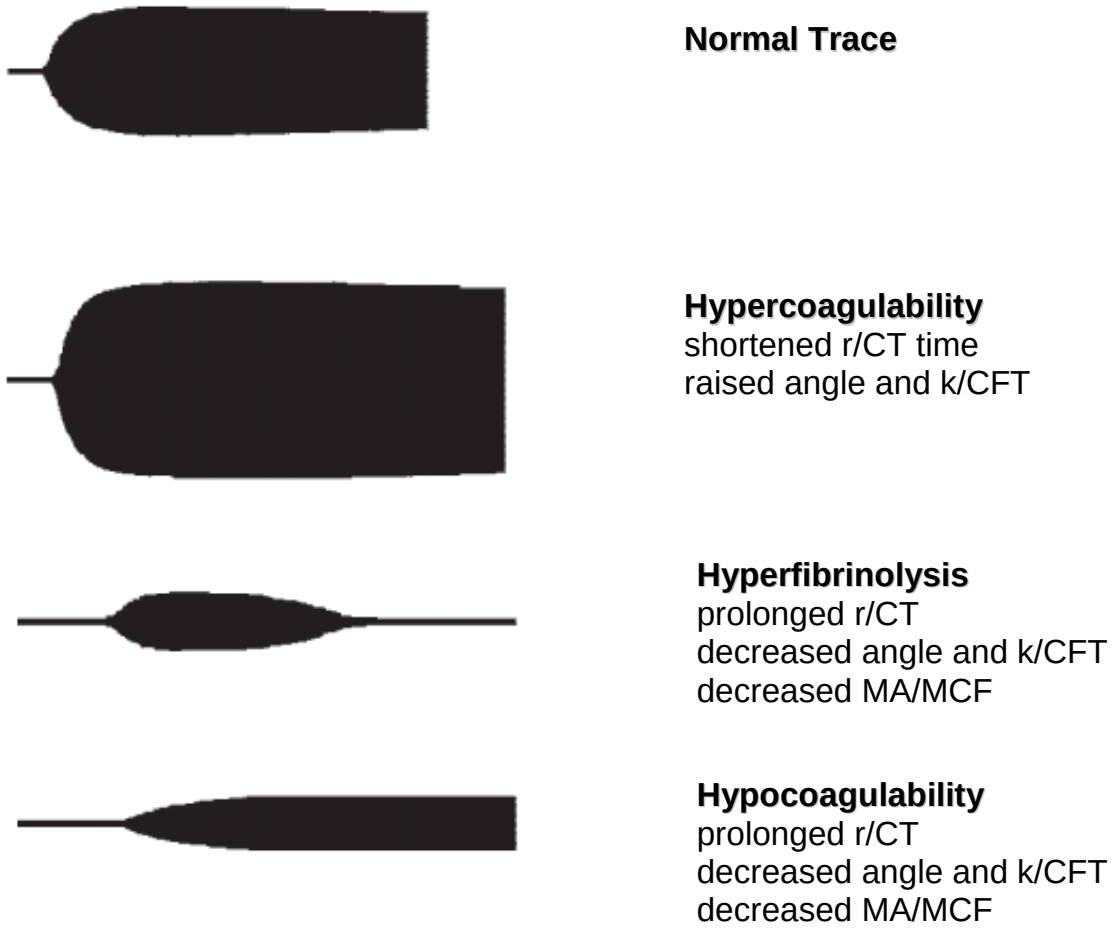


Figure 1.10 illustration of normal trace and abnormal traces generated during thromboelastography/thromboelastometry (adapted from Luddington, 2005)

1.9.2.2 Standardisation of the TEG assay

Lang and co-workers recently conducted comparison studies on reference ranges in this assay between centres (Lang *et al*, 2005). Previously, variability in results was a result of instrument performance and sample handling. Recent advances in technology and novel reagents have improved this greatly. Lang and his group showed that reagent-supported thromboelastography is reproducible and that the reference ranges were consistent between centres. This means that there can be standard ranges used in this assay for clinical use. Standardising the assay begins with standard reference ranges derived from

within each centre. Intra-centre reference ranges have been influenced by several factors such as citrate anticoagulants. This is because analysis of these samples is influenced by incomplete inhibition of the activation the cascade (Zambruni *et al*, 2004). The sample is usually rested for 30 minutes before it is analysed and as long as all the tests are performed in a standardised manner and processed in the same way, then reference ranges are appropriate for result assessment (Luddington *et al*, 2005).

One of the current modifications to the TEG assay is the use of low concentration TF to initiate coagulation. This technique has been applied to monitoring therapy such as that with rFVIIa (Sorensen *et al*, 2003 (**a**); Sorensen and Ingerslev, 2004 (**b**)). In addition to this, analysis of data derived from this application was also described where a velocity profile (maximum velocity, time to maximum velocity and area under the first derivative curve) was calculated from the 1st derivative parameters. All these new parameters have been applied in therapy response analysis (Sorensen *et al*, 2003; Sorensen and Ingerslev, 2004: Sorensen and Ingerslev, 2005). This application has extended to other global assays such as thrombin generation (Luddington and Baglin, 2004).

1.9.2.3 Evaluation of TEG against laboratory screening tests

Laboratory screening tests, such as plasma factor assays, provide an indication for a specific area of haemostasis like FVIII deficiency in haemophilia A. However, assays such as the TEG have further helped elucidate some factors

(such as platelets and plasma content) that affect the outcome and therefore interpretation of the result.

Reports have shown that correlations between TEG variables with laboratory screening assays such as MA and the fibrinogen concentration as well as platelet count in the normal controls and hypercoagulable individuals exist (Luddington, 2005). Other parameters include the APTT with the r/CT parameter and the lysis time with the euglobulin clot lysis time.

The TEG has played a role in the use of blood replacement products especially during and post hepatic and cardiac surgery. It has the ability to simulate haemostasis, a factor that has been adapted in order to improve and expand on the application of the assay. In the laboratory setting, anticoagulated samples are required for testing indicating a need for stricter interpretation of results. Currently, the TEG as a haemostatic reference has proved beneficial but there are still areas of improvement such as standardisation and optimisation of reagents used in the assay.

1.9.3 Calibrated Automated Thrombography

This method was developed by Hemker in 1986 based on the assumption that thrombin generated in tested plasma or whole blood was an accurate representation of the total combination of activities and concentrations of procoagulants and their anticoagulants. Simply, thrombin generation is measured after the addition of TF, phospholipids and calcium, when testing plasma, at fixed time intervals in a solution that contains a chromogenic

substrate. The rate of thrombin generation is displayed on a thrombin generation curve from which additional parameters may be calculated such as the lag time, peak height, time to peak and the area under the curve – Endogenous thrombin potential (ETP). The method has been modified over the years whereby, initially, a chromogenic substrate with slow reactivity can be used which allows for the release of para-nitroaniline by thrombin. Recently, fluorogenic substrates have been introduced to replace chromogenic substrates (Hemker, *et al*, 2000). With this, the fluorogenic signal is not disturbed by the turbidity from fibrin formation. This allowed plasma to be tested without defibrination as defibrination was required previously to maintain the transparency of the sample. This also meant that responses to therapy such as that with rFVIIa could be investigated in platelet rich plasma systems (Wegert *et al*, 2005). Results from this assay are still dependent on several variables such as TF concentration and phospholipids used to initiate the reaction. This assay has been noted to be useful in detecting deficiencies in factor deficiencies (FV, FVII, FVIII, FIX and FX) in platelet poor plasma but this assay can be used to monitor response to rFVIIa therapy in haemophilia.

1.10 RESEARCH AIM

Critically ill patients present a breakdown of their haemostatic balance that may eventually lead to multiple organ failure, without therapeutic intervention. There are several abnormalities in haemostasis of varying degree that develop in these patients, ranging from minor ones such as prolonged clotting times and isolated thrombocytopaenia to complex ones such as disseminated intravascular coagulation (DIC). There are several underlying causes that may be responsible for disturbed coagulation in critically ill patients, all which require specific therapeutic intervention.

This research project was designed as a preliminary study for analyses employing the use of global assays to analyse clotting in samples taken from ICU patients. The aims were to analyse the clotting profiles in samples taken from ICU patients with variable predisposing illnesses. Analysis was to be carried out on both whole blood and plasma samples using the thromboelastography (TEG) assay to analyse clotting status and the use the modified CLoFAL assay to analyse the rate of clot lysis. Clinical information on these patients was withheld and the samples anonymised to prevent bias in interpretation of the results obtained. These patients had been admitted to the ICU ward for over 1 week and therefore were considered to be very ill. Samples from two control groups were also used in the project, haemophiliac samples and samples taken from healthy individuals. Haemophiliac samples were taken at baseline level or in cases where the individual had factor concentrate administered, samples were taken 4 days after the administration of the last dose. Not only were the control groups used comparatively for the preliminary biochemical

analysis, but they were also used in analyses for sample responses to the pro-coagulant agent, activated recombinant FVII (chapter 3).

Before TEG and CLoFAL assay analysis was commenced, preliminary investigations were conducted to determine any biochemical alterations in coagulation in the ICU samples. This was carried out for each sample that was collected from the ICU patients. Alterations in coagulation were measured through parameters such as platelet counts, coagulation times, factor levels and fibrin degradation products. These data are shown in Section 3.2 and is discussed in Chapter 4.

Changes in coagulation can be measured by assaying parameters such as prolonged coagulation times, reduced levels of coagulation inhibitors, or increased fibrin degradation products. However, these are standard assays used within the laboratory to give a specified biochemical diagnosis but their outcome may not correlate with the clinical phenotype of the individual. Global assays are a benchmark for monitoring both pro-coagulant and anti-coagulant effects on haemostasis, be it from administered therapy or *in vivo* alterations. To date there is no assay that reflects the overall *in vivo* haemostatic profile. Global assays are currently used to highlight any changes in clinical phenotype and also monitor responses to therapy. The TEG assay in this project was used to measure the clot elasticity by using continuous graphical representation as the clot was formed. This assay displays an image, derived from calculations made during the formation of the clot by the thromboelastograph, which represent the firmness of the clot (Figure 1.8). The size and stability of the clot

formed was evaluated based on calculations obtained during physical formation of the clot. Based on the nature of the sample, variable traces (Figure 1.9) were observed and their parameters recorded and analysed.

Several groups have reported on the ability of rFVIIa to improve haemostasis, especially in haemophilia and other deficient disorders (Von Depka 2005; Lusher *et al*, 1998; Hedner and Ingerslev, 1999; Abshire and Kenet, 2004 (a); Gerotziafas *et al*, 2004; Almeida *et al*, 2003; He *et al*, 2005; Galan *et al*, 2003; Goodnough, 2004; Sorensen *et al*, 2003 (**a**); Sorensen and Ingerslev, 2004 (**b**); Sorensen *et al*, 2005 (**c**)). In addition to the above analyses, the TEG was used to observe the response to manipulation with infused rFVIIa in both the ICU samples and control samples. The response to rFVIIa was analysed in both whole blood and plasma samples.

Thrombin generation was carried out by a collegue, who was investigating thrombin generation in the same samples collected from the ICU patients. With his permission, this data was included and evaluated along side the finding from this project. This data is shown in Section 3.2.2.

In order to analyse clot stability within a plasma system, another assay was used. The modified CLoFAL assay derived from the methods published by Lisman et al, 2002 and Goldenberg et al, 2005 employs both clot formation and clot lysis. Because this assay quantitatively measures the rate at which the clot is formed or lysed, it was used to analyse the rate at which the formed fibrin clots within a plasma system where being cleared in the presence of a lysis trigger. The rationale behind

this analysis was that the lower the rate of lysis, the more stable the clot is. This analysis is highly influenced by the biochemical composition of the plasma but it also tells of the role the biochemical factor in stabilising the fibrin clot formed.

CHAPTER 2

MATERIALS and METHODS

2.1 CHEMICALS AND REAGENTS

All chemicals, reagents and supplies were purchased from the stated sources.

2.2 PATIENT SELECTION

The investigations in this project were carried out on whole blood and plasma taken from three defined groups; healthy control group (n=14), haemophiliac A patients (n=9) and Intensive care unit (ICU) patients (n=12). The haemophiliac patients are registered with the haemophilia care centre at Addenbrookes hospital while the ICU patients were enrolled from the intensive care unit at Addenbrookes hospital. The ICU patient group samples were obtained from a clinically diverse group of patients whose underlying conditions for being in the ICU varied among each patient. The reason for this unbiased selection was to assess the clotting profile in ICU patients as a whole rather than those with a particular associated disorder. Ethical approval is included in Appendix I. Only samples at baseline factor levels were taken from the haemophiliac subjects. Alternatively, these haemophiliac subjects had to be free of replacement factor for a period of 72 hours or more.

ICU samples were obtained from patients that indicated fragility in their haemostatic ability. This involved a range of patients, from those with organ dysfunction to those who had undergone surgery during their duration at the hospital. Confirmatory status of haemostatic profile was elucidated by analysis using haemostasis screening tests; as explained in Section 3.2.

2.3 SAMPLE ACQUISITION AND PREPARATION

Venous blood samples were drawn from the arm by a phlebotomist into 3.8% (w/v) tri-sodium citrate (0.109M) and the blood reconstituted 9 part volumes of blood to 1 part volume of citrate, in a Starstedt blood collection bottle (Starstedt, Numberecht, Germany). These samples were centrifuged at 3000g for 10 minutes and the platelet poor plasma aliquoted into 2ml capacity cryovials and frozen at -80°c for subsequent testing.

2.4 REAGENT PREPARATION

2.4.1 Phospholipid preparation

Extrusion device (Avestin, Ottawa, Canada)

Polycarbonate filters (Glen Creston, Middlesex, UK)

Synthetic phospholipids in chloroform (Avanti polar lipids, Alabama, USA)

Synthetic phospholipids in chloroform (Avanti polar lipids, Alabama, USA) were prepared as described by Falls *et al* (2000). The lipids were combined in the required proportions of phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The lipids were then dried under nitrogen at 45˚C for 30 minutes and there after resuspended in HEPES buffer (50mM HEPES, 145mM NaCl, pH 7.1). This mixture was passed through the extrusion device using a 100nm diameter polycarbonate filter. Another extrusion step was run using a 50nm diameter

polycarbonate filter and the resultant vesicles stored at 4˚C until later use. The final ratio of the phospholipid components was as follows: PS (20%)/PE (20%)/PC (60%).

2.4.2 Tissue factor preparation

Commercial thromboplastin tissue factor, Innovin, Sysmex UK Ltd, Milton Keynes, UK

Working buffer: Thrombogram buffer

Recombinant human tissue factor (TF), Innovin®, was purchased from Sysmex UK Ltd, Milton Keynes, UK.

Tissue factor was diluted to the required working concentration using working buffer. Working buffer contained 20mM HEPES, 140mM NaCl, 5mg/ml BSA pH 7.4

2.4.3 Tissue plasminogen activator (tpa) preparation

Tissue plasminogen activator (2-chain tpa) , American Diagnostica Inc, Stamford, Connecticut, USA.

HEPES buffer (3.5mM KCL, 137mM NaCl, 25mM HEPES, 3mM $CaCl_2$, pH 7.4)

Tissue plasminogen activator (tpa) was purchased from American Diagnostica Inc, Stamford, Connecticut, USA. HEPES buffer was used to dilute a series of tpa concentrations as required.

2.4.4 Recombinant human FVIIa preparation

Recombinant activated factor seven (NovoSeven, eptacog alfa {activated}), Novo Nordisk, Bagsveard, Denmark

NovoSeven was a kind gift from the Haemophilia centre at Addenbrookes NHS Hospital. Each vial contained 2.4mg/ml of lyophilised rFVIIa. On reconstitution, with sterile water, each vial contained 2.4 mg/ml of novoseven. The reconstituted product contains no preservatives. NovoSeven contains trace amounts of proteins derived from the manufacturing and purification processes such as mouse IgG (maximum of 1.2 ng/mg), bovine IgG (maximum of 30 ng/mg), and protein from BHK-cells and media (maximum of 19 ng/mg).

On reconstitution, rFVIIa was aliquoted and frozen at -80°C for future use. Stock solution of 10mg/ml, 1,86mg/ml and 0.6ug/ml were reconstituted and stored at -80°C. Aliquots were thawed at room temperature for 5 minutes and immediately placed on ice and were used within 1 hour after thawing occurred.

2.4.5 Calcium chloride

Calcium chloride (volumetric solution of 1M), BDH, Poole, England

Patient samples were recalcified by adding 0.2M $CaCl_2$ (reconstituted from 1M $CaCl_2$ in 25mM HEPES buffer containing 140mM NaCl, p.H 7.4)

2.4.7 Multi-channel discrete analyser (MDA) series analyser reagents.

Devices and utilities used included:

- ***a.*** *MDA series coagulation analyser (Trinity Biotech, Sulhamstead, Theale, UK)*
- ***b.*** *Disposable cuvettes (Trinity Biotech, Sulhamstead, Theale, UK)*

c. *Imidazole buffer, (Trinity Biotech, Sulhamstead, Theale, UK)*

d. *Platelin LS and CaCl reagent (Trinity Biotech, Sulhamstead, Theale, UK)* was used in APTT-based assays in plasma. The assay works by employing a phospholipid reagent with activators as well as a calcium chloride reagent

e. *MDA D-dimer kit (Trinity Biotech, Sulhamstead, Theale, UK)* assay is based on a homogenous latex particle based immunoassay for the quantification of cross-linked fibrin degradation products containing the D-dimer domain in plasma.

f. *MDA Fibriquik kit (Trinity Biotech, Sulhamstead, Theale, UK)* is bovine thrombin reagent for the use in the quantitative determination of fibrinogen levels.

g. *Simplastin rHuTF (Trinity Biotech, Sulhamstead, Theale, UK)* is a tissue thromboplastin reagent derived from cultured human cells used to determine the prothrombin time in plasma.

h. *Factor deficient plasma (Trinity Biotech, Sulhamstead, Theale, UK). T*hese are intended for the quantification of respective factors in patients suspected of diminished or absent coagulation protein levels. [Levels analysed included FII, FV, FVII, FVIII and FX]

i. *Reference material (Technoclone, Vienna, Austria)*

j. *In-house normal and abnormal plasma reference*

METHODS

2.5 FULL BLOOD COUNT (FBC)

Beckman-Coulter analyser, High Wycombe, Buckinghamshire, UK

Diluents and Reagent kit solution, Beckman-Coulter, California, USA

Full blood count tests were carried out on the Beckman coulter analyser. The main parameters of this test include white cell count, haemoglobin estimation, red cell count, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration and platelet count. The FBC is mainly used as a screening test to indicate levels of anaemia and infection. The platelet count is critical in the haemostatic process as it is an indicator of thrombocytopaenia.

Assay method:

Venous blood samples were obtained from patients using recommended the protocol into 1.6Mg/ml EDTA in a S-monovette Starstedt collection bottle (Starstedt, Numberecht, Germany) and were handled and processed according to the recommended laboratory protocol (sufficiency, clots and correct labelling). Once analysed, the samples were authorised and results transferred to the laboratory information systems.

Reference ranges and units (age and sex-linked) were defined on the laboratory information systems (e.g. platelet count = 150-450x10^9/L).

2.6 THROMBOELASTOMETRY

ROTEM (Pentapharm GmbH, Munich, Germany) {Figure 2.1}

Disposable cups and pins (Pentapharm GmbH, Munich, Germany)

Relipidated tissue factor

0.2M CaCl

Principle of the method:

Blood is added into disposable cuvette in heated cuvette holder and the disposable pin (sensor) is fixed on tip of rotating axis as shown in figure 2.1. The axis is guided by high precision ball bearing system and the axis rotates back and forth. This axis is connected with spring for measurement of elasticity. The exact position of the axis is detected by reflection of light on small mirror on axis. The data is then obtained and analysed on computer. The loss of the elasticity when the sample clots leads to a change in the rotation of the axis and so this is represented as a visual trace called a thromboelastogram (Figure 1.10).

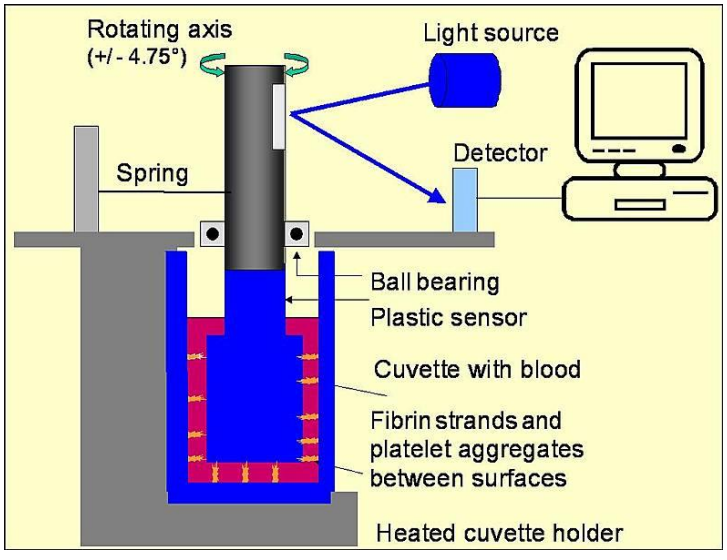


Figure 2.1 Principle of thromboelastometry (Figure obtained from www.pentapharm.de)

Assay method:

Whole Blood

Instrument preparation

The analyser was switched on and allowed to warm up to 37°C. Cup carriers, to be used for analysis, were carefully removed and placed on the heated area at the front of the instrument to maintain the carrier temperature at 37°C. Cup and pin were taken from the box, ensuring that only the exterior of the cup surface was touched. If the pin was touched, then both the cup and pin were discarded. Holding the outside of the cup, the pin on the carrier spike for the channel to be used was located. The cup was placed in the cup carrier and sample processing and analysis commenced.

Performing the analysis

The channel number and tests to be used were selected from the software. Using the electronic pipette, the instructions on screen were followed for sample and reagent delivery (with whole blood, the sample must be fully mixed by gentle inversion and all testing preformed within 2 hours post venapuncture). A pronounced variance in the TEG parameters is obtained within the first 30 minutes after blood is taken, all samples were rested for 30 minutes before analysis (Sorensen *et al*, 2003)The cup was carefully slid into position, ensuring that the two pins at the back secured the cup magnetically. Analyses were carried out for 45-60 minutes.

Plasma

A volume of 20µl of relipidated tissue factor was added to the cup followed by 20µl of $CaCl_2$. After the addition of 300µl of test sample, the cup was adjusted on to the pin and the thromboelastograph trace initiated as explained above.

Parameters measured and reference ranges

Parameters measured are shown in Figure below (Figure 2.2). The numerical data can be compared to the provisional reference ranges.

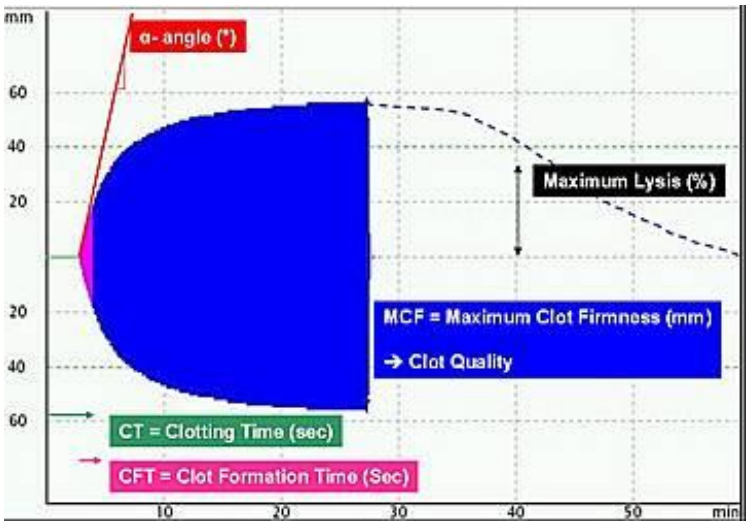


Figure 2.2 Thromboelastograph trace (Figure obtained from www.pentapharm.de)

Parameter	Range	Units
CT	**420-750**	**sec**
CFT	**75-420**	**sec**
Angle	**30-63**	**degrees**
MCF	**41-63**	**mm**
ML	**1-10**	**%**

Table 1.2 Reference ranges and units (Provisional ranges n=8) (CT, Clotting time; CFT, Clot formation time; MCF, Maximum clot formation; ML, maximum lysis; LI30, Lysis index 30).

2.7 CALIBRATED AUTOMATED THROMBIN GENERATION ASSAY

Fluoroscan Ascent (Thermolabsystems OY, Helsinki, Finland)

Thrombinoscope software (Synapse BV, Maastricht, Netherlands)

Microplates; 96 wells (Greiner Bio-one Ltd, Storehouse, UK)

Fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Dubendorf, Switzeland)

Phopsholipid vesicales (section 2.4.1)

Recombinant human TF, innovin (Sysmex Ltd, Milton Keynes, UK)

Dimethyl sulphoxide (DMSO) (Sigma Aldrich Ltd, Poole, UK)

Calcium chloride (BDH Ltd, Poole, UK)

Bovine serum Albumin (BSA) (Sigma Aldrich Ltd, Poole, UK)

20mM HEPES buffer

Thrombin calibrator (Synapse BV, Maastricht, Netherlands)

This assay (Hemker et al, 2003) was conducted with 20µl of buffer (20mM HEPES buffer, 140mM NaCl, 5mg/ml BSA, pH7.4) containing lapidated TF which was added to the microplate and 80µl of sample added to these wells. Substrate reagent, 20µl, (2mM BSA, pH 7.4) 20mM HEPES, 60mg/ml BSA, pH 7.4) and 100µl 1M $CaCl_2$, 25µl of fluorochrome in 100mM DMSO were added and the reaction monitored using the thrombinoscope software. Contact factor activation was eliminated using corn trypsin inhibitor (CTI). Thrombin generation was carried out by a colleague, who was investigating thrombin generation in the same samples collected from the ICU patients as part of another on-going project. This data is shown in Section 3.2.2, with his permission.

2.8 COAGULATION LABORATORY ANALYSIS

The multi-channel discrete analyser (MDA) series analyser was used to test plasma components of citrated samples taken from ICU patients

2.8.1 MDA prothrombin time (PT)

MDA series coagulation analyser (Trinity Biotech, Sulhamstead, Theale, UK)

Disposable cuvettes (Trinity Biotech, Sulhamstead, Theale, UK)

Imidazole buffer, (Trinity Biotech, Sulhamstead, Theale, UK)

Simplastin rHuTF (Trinity Biotech, Sulhamstead, Theale, UK)

Addition of tissue thromboplastin, phospholipid and calcium ions to citrated plasma induces clotting, based on the principle shown in Figure 2.3. The clotting time depends upon the levels of FII, FV, FVII and FX (these are factors that constitute the extrinsic pathway/tissue factor pathway). The laboratory reference range was 11-14 seconds (mean normalised PT = 12.2 seconds)

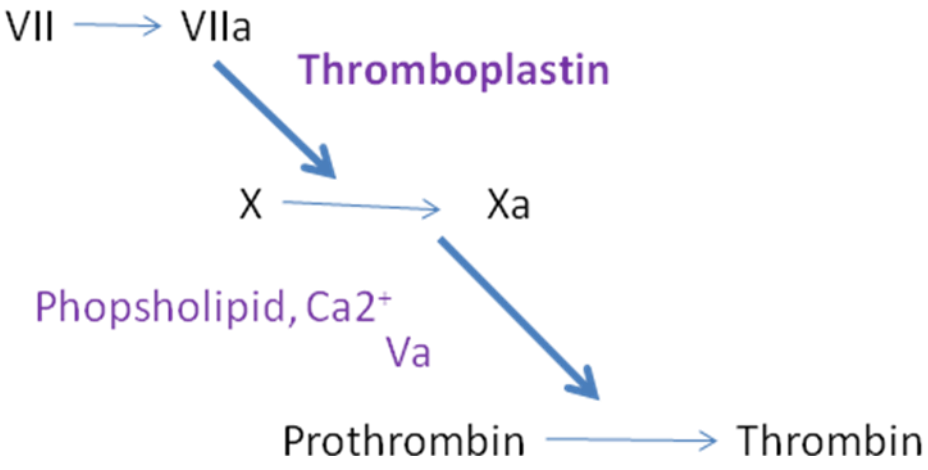


Figure 2.3 Principle of the PT assay on the MDA

2.8.2 MDA Activated partial thromboplastin time (APTT)

MDA series coagulation analyser (Trinity Biotech, Sulhamstead, Theale, UK)

Disposable cuvettes (Trinity Biotech, Sulhamstead, Theale, UK)

Imidazole buffer, (Trinity Biotech, Sulhamstead, Theale, UK)

Platelin LS and CaCl reagent (Trinity Biotech, Sulhamstead, Theale, UK)

In order to activate the contact factors in the intrinsic pathway, contact activation is used and this is facilitated by the large anionic surface area of the added micronized silica. On addition calcium and phospholipid, the time for clot formation is dependent on the presence of clotting factors in the intrinsic pathway as shown in Figure 2.4. The Laboratory reference range 22.5-34.5 seconds (mean normalised APTT = 28.5 seconds.

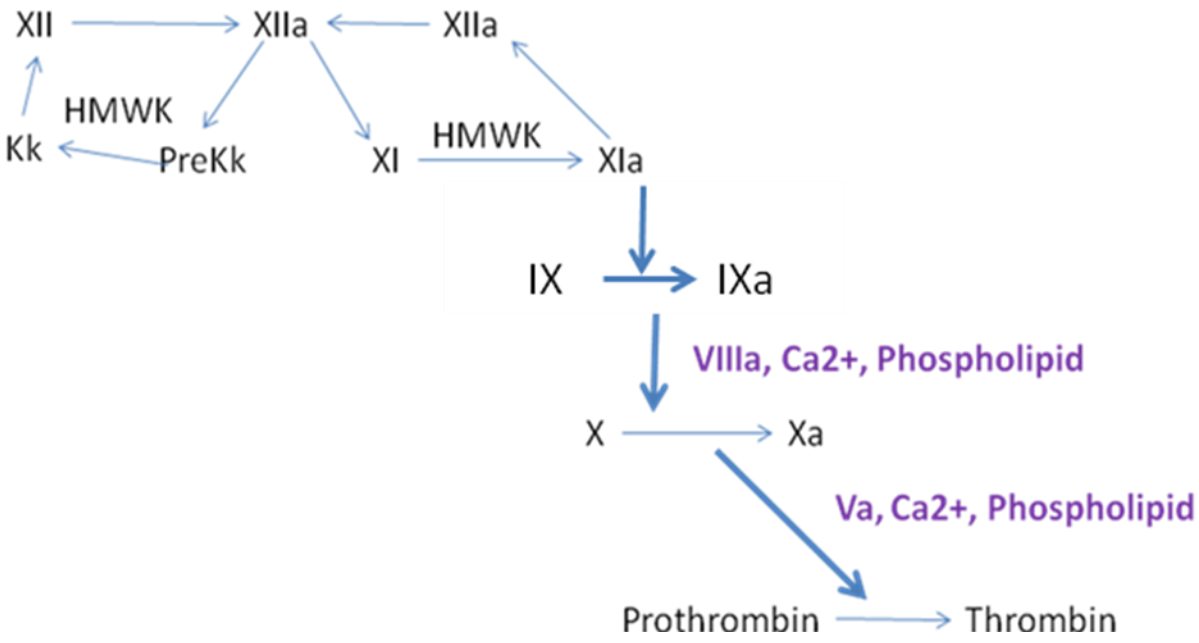


Figure 2.4 principle of the APTT assay on the MDA

2.8.3 MDA Clauss fibrinogen assay

MDA series coagulation analyser (Trinity Biotech, Sulhamstead, Theale, UK)

Disposable cuvettes (Trinity Biotech, Sulhamstead, Theale, UK)

Imidazole buffer, (Trinity Biotech, Sulhamstead, Theale, UK)

MDA Fibriquik kit (Trinity Biotech, Sulhamstead, Theale, UK)

This automated fibrinogen test relies on the principle that fibrinogen is transformed into fibrin in the presence of excess thrombin. Thrombin is incubated with diluted patient plasma and the clotting time is inversely proportional to the concentration of fibrinogen concentration in the sample. The concentration of fibrinogen present in the sample is calculated from a calibration curve derived by the software. Reference range 1.5-4.5g/L (mean $\pm$ 2SD, n=40).

2.8.4 MDA D-Dimer assay

MDA series coagulation analyser (Trinity Biotech, Sulhamstead, Theale, UK)

Disposable cuvettes (Trinity Biotech, Sulhamstead, Theale, UK)

Imidazole buffer, (Trinity Biotech, Sulhamstead, Theale, UK)

MDA D-dimer kit (Trinity Biotech, Sulhamstead, Theale, UK

This is a latex based immunoassay that measures D-dimer antigen present in plasma as a result of fibrin clot degradation by plasmin. Elevated levels are associated with secondary fibrinolysis following clot formation. Latex particles coated with a mouse monoclonal D-dimer antibody are added to diluted plasma which allows the particles to agglutinate in the presence of D-dimers. There is a positive correlation of the D-dimer concentration to the rate of agglutination of the particles. D-dimer levels are

measured as µg FEU/L (fibrinogen equivalent units per litre). Normal range is <500 µg FEU/L.

2.8.5 MDA PT-based assays for FII, FV. FVII and FX

MDA series coagulation analyser (Trinity Biotech, Sulhamstead, Theale, UK)

Disposable cuvettes (Trinity Biotech, Sulhamstead, Theale, UK)

Imidazole buffer, (Trinity Biotech, Sulhamstead, Theale, UK)

Platelin LS and CaCl reagent (Trinity Biotech, Sulhamstead, Theale, UK)

Simplastin rHuTF (Trinity Biotech, Sulhamstead, Theale, UK)

Factor deficient plasma (Trinity Biotech, Sulhamstead, Theale, UK)

This is a one-stage assay based on the PT that relies on the patient sample plasma correcting the clotting time of the kit's deficient plasma. By adding the respective deficient substrate plasma allows for the detection of any correction that may occur as a result of adding patient sample plasma. The prolongation of the test time is proportional to the respective factor level present in the patient sample. Sample factor levels are calculated from a standard reference curve derived from dilutions of the commercial normal reference plasma. Results are reported as a percentage of the activity by extrapolation from the reference curve.

Reference ranges:
FII = 50-200 IU/dL (Dacie and Lewis, 2006)
FV = 50-200 IU/dL (Dacie and Lewis, 2006)
FVII = 63-171 iu/dL (n=20, sept. 1996)
FX = 59-169 IU/dL (n=20, sept, 1996)

2.8.6 MDA APTT-based assays for FVIII

MDA series coagulation analyser (Trinity Biotech, Sulhamstead, Theale, UK)

Disposable cuvettes (Trinity Biotech, Sulhamstead, Theale, UK)

Imidazole buffer, (Trinity Biotech, Sulhamstead, Theale, UK)

Platelin LS and CaCl reagent (Trinity Biotech, Sulhamstead, Theale, UK)

Simplastin rHuTF (Trinity Biotech, Sulhamstead, Theale, UK)

Factor deficient plasma (Trinity Biotech, Sulhamstead, Theale, UK)

This is a one-stage assay based on the APTT that relies on the patient sample plasma correcting the clotting time of the kit's deficient plasma. By adding the respective deficient substrate plasma allows for the detection of any correction that may occur as a result of adding patient sample plasma. The prolongation of the test time is proportional to the respective factor level present in the patient sample. Sample factor levels are calculated from a standard reference curve derived from dilutions of the commercial normal reference plasma. Results are reported as a percentage of the activity by extrapolation from the reference curve.

Reference range:

FVIII = 48-138 IU/dL (n=20, sept. 1996)

2.9 CLOT LYSIS ANALYSIS

2.9.1 Clot lysis assay

Phospholipid, 100uM

Tissue factor, 9.3pM

Tpa, 5µg/ml

$CaCl_2$, 0.2mM

Multiscan Ascent microplate reader (Thermo Labsystems, Basingstoke, UK)

Microtitre plates (96 wells) (Bachem UK Ltd, St. Helens, UK)

This method of investigation was based on the procedure used by Lisman et al, 2002 and Goldenberg et al, 2005. Reagent concentrations were optimised for this assay as described in Chapter 3. Platelet poor plasma samples were thawed at 37˚C and placed on ice until the time of assay but did not exceed 25 minutes incubation on ice. Reaction lysis buffer was prepared from tpa (3µg/ml), phospholipid (100µM), $CaCl_2$ (0.2M) and tissue factor (9.3ρM) all which were added to buffer and to give the following final concentrations (tpa; 375ng/ml, PL; 12.5µM, $CaCl_2$; 2.5mM and TF; 1.1625ρM) after addition of the reactant solution to plasma samples.

For each patient sample tested, 85µL of freeze-thawed plasma was dispensed into each of 3 wells of a 96-well plate (with reagent blank in the fourth) and 85µL of reactant solution was added simultaneously to the remaining wells. The plate was then placed in a microplate reader and dual kinetic absorbance measurements at 405nm wavelength, at 45 second intervals, for 5 hours were recorded. The spectrometer

interfaces with a computer in that all its operations such as continuous analysis of absorbance using the software can be automated. Absorbance data was blanked by the reagent in order to eliminate artefact changes in baseline absorbance due to lipaemic plasma. Blanked absorbance data was then averaged at each time point for the triplicate wells.

Data retrieved from this experiment was based on the increase in light absorbance, which is proportionate to the maximum amplitude. Likewise, the decline in absorbance is relative time taken for the maximum amplitude to reduce to its lowest level. The rate of clot formation is therefore represented by the phase that reaches maximum amplitude and the rate of clot lysis is represented by the phase that reaches minimum amplitude.

2.10 STATISTICAL ANALYSIS

The non-parametric Kruskal-Wallis test was used to test the variation between groups and one-way analysis of variance (ANOVA) was used for changes before and after addition of rFVIIa. GraphPad prism statistical software package (GraphPad Software, California, USA) was used for statistical analysis. $P<0.05$ was considered to be statistically significant.

CHAPTER 3

RESULTS

3.1 ANALYSIS OF THE CLOTTING PROFILE OF WHOLE BLOOD

3.1.1 Platelet counts among patient groups

As part of the preliminary investigations, platelet counts were measured in whole blood samples obtained for each patient group. Platelet counts ranged from

118-305.5x10^9/L in Healthy volunteers (n=14),

143.3-311.0 x10^9/L in haemophiliacs (n=7) and

12.20-451.1 x10^9/L in ICU (n=12) samples.

The group platelet count means were 228.9±20.10 x10^9/L (healthy volunteers), 224.9±20.19 x10^9/L (haemophiliacs) and 177.5±38.59 x10^9/L (ICU) as shown in Figure 3.1. No statistical significance was observed between the healthy volunteers, haemophiliac and ICU sample group platelet counts.

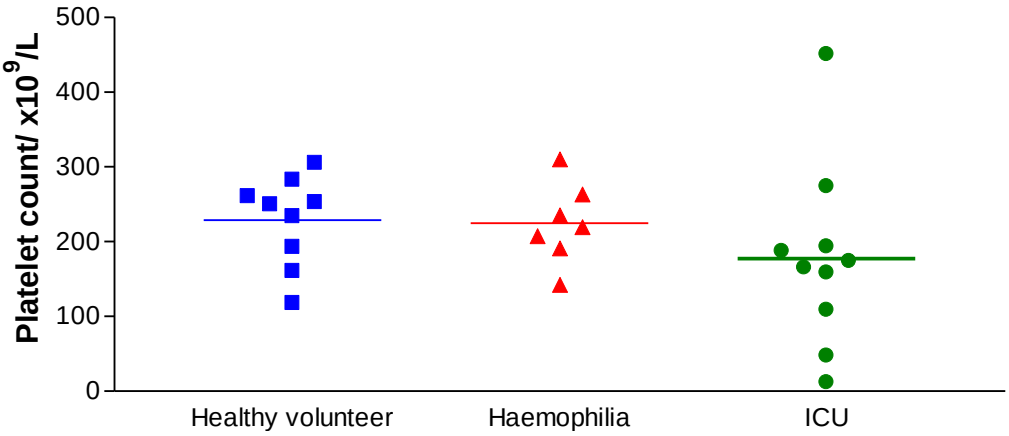


Figure 3.1 Platelet counts for respective patient groups for whole blood samples collected. Healthy volunteer controls (n=14), haemophiliacs (n=7) and ICU (n=12)

CONCLUSION: The variation within these counts is more noticeable in the ICU patient group (showing a range of 12.20 - 451.1 x10^9/L) than the healthy volunteer or

haemophiliac group. The underlying pathology in these individuals could play a role in the platelet levels noted in this ICU group. Other coagulation data that further support this deduction is shown below.

3.1.2 Whole blood (WB) clotting profile among patient groups using the thromboelastogram

Prior to the investigation of thrombin production and fibrin formation in a plasma system, whole blood profiles of the samples obtained from each individual within the respective patient group were analysed. Previous investigations have shown that platelet counts affect the rate of clot formation and the size of the clot formed (i.e. the maximum amplitude of the clot as measured on the ROTEM). In addition, the presene of rFVIIa improves the time and rate of clot formation (Gerotziafas *et al*, 2004, Gabriel *et al*, 2004, Sorensen and Ingerslev, 2004).

The aim of this investigation was to initially assess the *ex vivo* clotting profile of each sample taken from each individual within the patient group. In addition, later on the responses of these samples to rFVIIa was also investigated.

Figure 1.12 shows a traditional thromboelastograph trace with an illustration of the dynamic profile after a raw signal has been applied to a sample for clotting to initiate. This pattern was analysed during the thromboelastographic investigation of coagulation in normal, haemophiliac and ICU whole blood samples. The clotting time (CT) corresponds to the initiation phase of clot formation. The maximum clot firmness (MCF) is the measure of the impact on clot elasticity of the platelet count

and fibrinogen concentration and the clot angle (α) correspond to the size of the clot formed and the rate at which it progresses.

ROTEM was carried out on native blood samples (Figure 3.2A) and subsequently, to a second aliquot of that sample to which, 5µl of (giving a final concentration 8.82µg/ml) rFVIIa was added (Figure 3.2B). In all samples, a profile of real time continuous plasma clot formation was recorded by a ROTEM analyser as explained in Section 2.6. Coagulation was initiated by addition of 20µl of 0.2M $CaCl_2$ and 20µl of 1: 1000 TF and all analyses were allowed to proceed for 45-60 minutes.

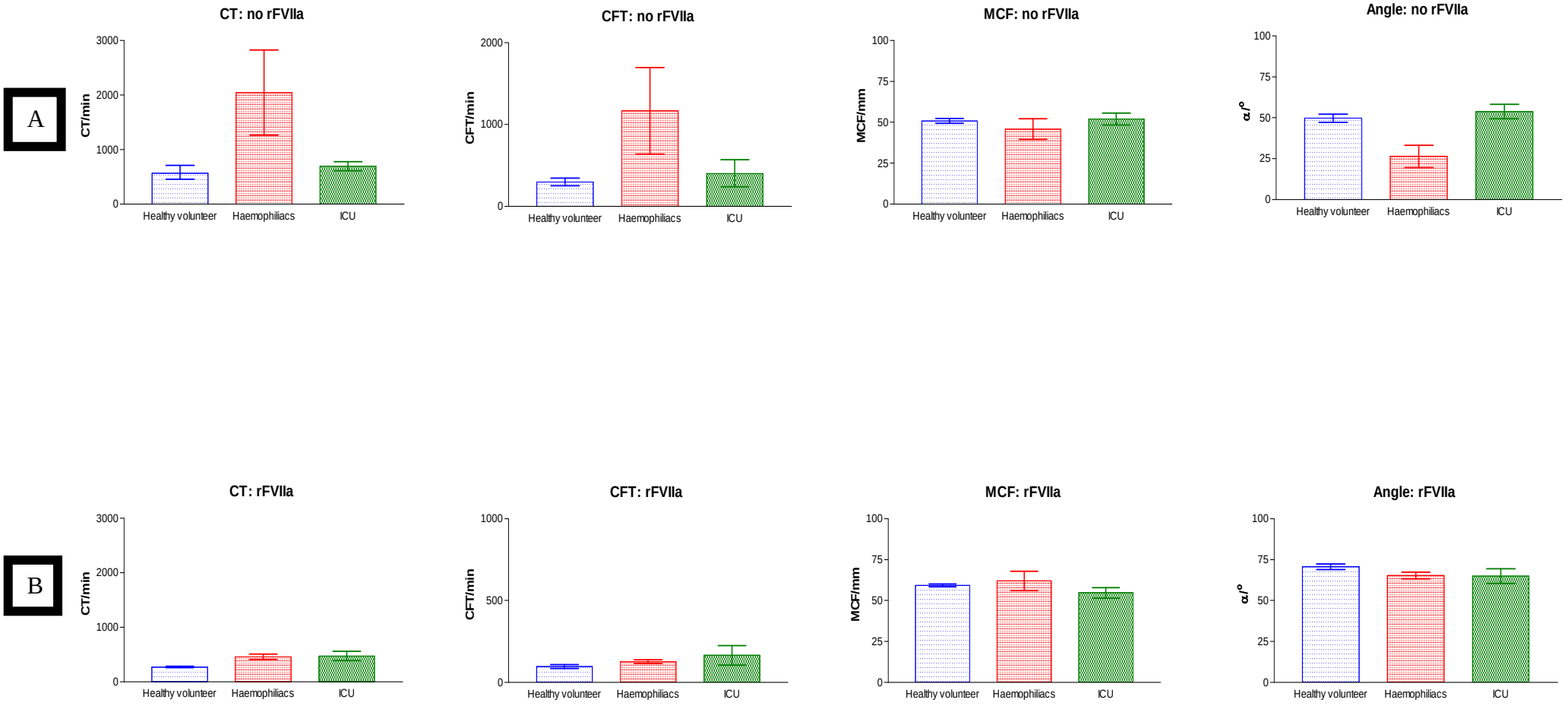


Figure 3.2 ROTEM parameters on whole blood recorded on healthy volunteers, haemophiliac and ICU samples. Graphs show clot profiles pre (A) and post (B) 8.82µg/ml infusion of rFVIIa. Data is represented as the mean ±SEM. CT, clotting time; CFT, clot formation time; MCF, Maximum clot formation; α, clot angle; rFVIIa, recombinant activated FVII

CONCLUSION: The results presented in Figure 3.2**A** show thromboelastograph analysis carried out on native whole blood activated by just the addition of $CaCl_2$. These results show a marked difference in clot formation especially within the haemophiliac group. The CT and CFT within the haemophiliac group was characterised by prolonged clotting times and slow progression of clot formation in comparison to the healthy volunteer group and ICU group. The clotting times in the healthy volunteer and ICU whole blood were similar. Non-parametric analysis (Kruskal Wallis test) was carried out to analyse variation between the groups in the absence of rFVIIa and showed that inter-variation among the groups was as follows: CT, p=0.052; CFT, p=0.0105; α, p=0.0032; there was no statistical variation in the MCF among these groups. Table 3.1 shows the 2 samples from the haemophilia group and the ICU group that failed to be activated upon addition of $CaCl_2$.

	Normal	Haemophiliac	ICU
CT (sec); NR:450-690sec			
Range	457 – 708 ***(215-344)***	635.0 – 5529 ***(347-689)***	420 – 1370 ***(264-1034)***
Mean ± SEM	563.5+22.26 ***(271.2±13.10)***	2044+780.5 ***(458.7±17.20)***	696.4+83.35 ***(474.9±85.46)***
CFT (sec); NR:120-450sec			
Range	146 – 782 ***(56-181)***	391 – 3720 ***(89-160)***	111 – 2142 ***(63-691)***
Mean ± SEM	299.5+47.50 ***(96.73±11.69)***	1168+528.5 ***(127.3±11.77)***	403+166.0 ***(165.4±59.00)***
MCF (mm); NR:34-46mm			
Range	43 – 61 ***(53-62)***	21 – 66 ***(51-89)***	26 – 63 ***(32-63)***
Mean ± SEM	50.85+1.467 ***(59.27±0.9351)***	95.33+6.258 ***(62±5.888)***	52.00+3.601 ***(54±3.194)***
Clot angle, α (˚); NR:36-48°			
Range	41.50 – 63 ***(60-78)***	0 – 35 ***(60-72)***	0 – 68 ***(26-77)***
Mean ± SEM	49.67+2.652 ***(70.64±1.760)***	26.40+6.779 ***(65.33±2.076)***	53.82+4.350 ***(65±4.575)***

Table 3.1 TEG parameters on native whole blood samples taken from healthy volunteers, haemophiliac and ICU samples; unbold Figures. TEG parameters on native whole blood samples from each respective group with the addition of rFVIIa; bold Figures in brackets. (The normal ranges (NR) are TEG manufacturer normal ranges.) [n=9, n=7 and n=10 respectively] CT, clotting time; CFT, clot formation time; MCF, Maximum clot formation; α, clot angle; rFVIIa, recombinant activated FVII

However, following the addition of 5µl of rFVIIa, the dynamics of clot formation shifted as shown in the bottom row in Figure 3.2 marked with the **B**. The greatest response to rFVIIa in respect to the ROTEM parameters was predominantly noted within the haemophiliac group (Table 3.2). Figure 3.2B shows thromboelastograph analysis of clot formation in whole blood activated with $CaCl_2$ in the presence of rFVIIa. The addition rFVIIa normalised all four parameters analysed using this assay in all three sample groups. Statistical analysis showed that there was no variation between the respective study group, in all four parameter, after the addition of rFVIIa ($p>0.05$). Within each study group, the most noticeable changes were noted in the CT and the CFT parameters.

	CT (decrease)	**CFT (decrease)**	**MCF(increase)**	**α(increase)**
Normal	52%	62%	15%	39%
Haemophiliac	80%	84%	21%	76%
ICU	33%	57%	0.09%	26%

Table 3.2 Percentage change in parameters after 5µl of 8.82µg/ml rFVIIa infusion into each respective group sample. CT, clotting time; CFT, clot formation time; MCF, Maximum clot formation; α, clot angle; rFVIIa, recombinant activated FVII

CONCLUSION: The initiation and propagation stages of thromboelastometry trace recording before the *ex vivo* addition of rFVIIa in each of the patients is most marked in the haemophiliac samples compared to samples from healthy volunteers and ICU samples. This might be attributed to the fact that intrinsic factors (FVIII and FIX) are deficient in the haemophiliac group whereas some of the results observed in the ICU group could be due to an acute phase response in these patients (see Section 3.2.1). This is discussed further with other supportive evidence below.

3.2 PLASMA SAMPLE ANALYSIS

3.2.1 Routine coagulation tests and coagulation factor levels in the ICU group

Although the ROTEM, as a global assay, proves a reliable indicator of the clotting status, more specific tests were performed to support the results obtained from the ROTEM assay. The strength of a Table clot is largely influenced by the presence of platelets in the whole blood system but in the plasma system this is comparable with fibrin formation.

Conventionally, clotting screen tests (PT and APTT) are carried out when a haemorrhagic or thrombotic disorder is suspected. The APTT is prolonged when there is a deficiency of one or more of the intrinsic factors or when fibrinogen levels in blood are decreased. Other circumstances where the APTT may be prolonged include the presence of specific inhibitors to circulating factors, non-specific inhibitors like lupus anticoagulant, interfering substances like fibrin degradation products or paraproteins. When both the APTT and the PT are prolonged and other factors like liver disease, uraemia, disseminated intravascular coagulopathy, oral anticoagulation or vitamin K deficiency have been excluded, then a single-factor deficiency can be suspected. A prolonged PT in conjunction with other tests is usually consistent with FVII deficiency and subsequently, diagnosis of a single factor deficiency is carried out using a one-stage PT-based assay.

The aim of this investigation was to assess the coagulation profile of the ICU group samples within a plasma system. Screening tests and single factor tests were

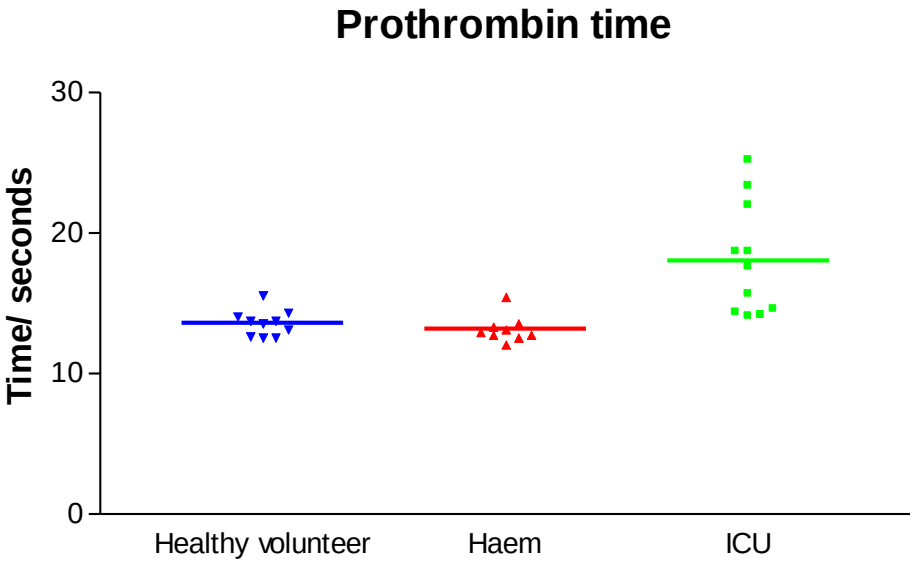
performed on the plasma samples as outlined in Section 2.7 (see Appendix i and ii). Factor assay reference ranges were standardised in-house, or in accordance with Dacie and Lewis, 2006 or commercially (*Trinity Biotech, Sulhamstead, Theale, UK).*

Coagulation tests carried out on ICU samples are shown in Figures 3.3a-d. In addition healthy volunteers and haemophilia samples were also analysed. A summary of this data is shown in Table 3.3 below.

Factor (RR)	Range	Median	Mean±SEM
DD (<500 FEU)	836.6 – 11,020	1845	3673 ± 942.2
Fbn (1.5-4.0 g/dL)	1.06-6.29	3.07	3.166 ± 0.43
PT (11-14 sec)	14.1 – 25.20	17.6	18.05 ± 1.197
APTT (24.5-34.5sec)	26.7 – 83.5	46.20	45.54 ± 4.967
FII (70-100 IU/dL)	27-79	46.5	45.83 ± 4.474
FV (70-150IU/dL)	23-160	61.5	71.83 ± 1.64
FVII (70-150IU/dL)	11-83	50	50.25 ± 7.22
FVIII (50-150IU/dL)	92 - 277	165	173.5 ± 18.20
FX (70-150IU/dL)	33 - 123	56	57.92 ± 6.980

Table 3.3 Summary of results derived from coagulation factor assays in ICU plasma samples. RR, Reference range; DD, d-dimer; Fbg, Fibrinogen; PT, Prothrombin time; APTT, Activated partial thromboplastine time; FII,V,VII,VIII and X, Factor II,V, VII, VIII and X

Non-parametric statistical analysis on the ICU patient group data showed statistical variation in comparison to the healthy volunteer group when data from coagulation factor assays was analysed (PT, p=0.0002; APTT, p=0.0004; FII, p=0.0001; FVII, p=0.0044; FVIII, =0.0001; FX, p=0.0005). The D-Dimer levels were only tested in the ICU patient group. The D-Dimer levels in the ICU samples were elevated above the reference range for the assay (RR = >500g/L FEU {fibrinogen equivalent units}).



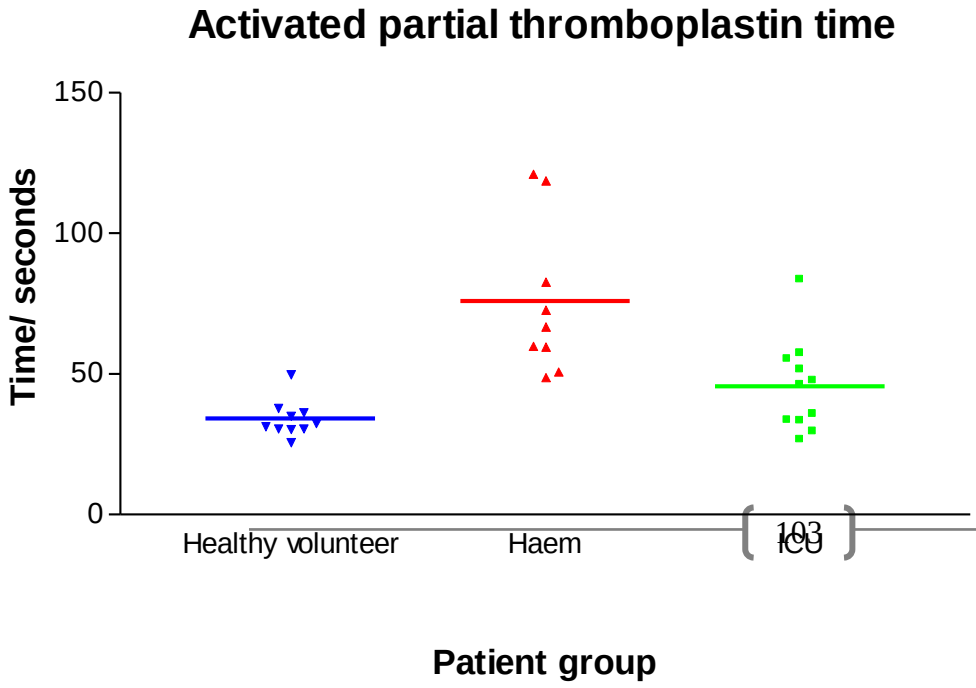
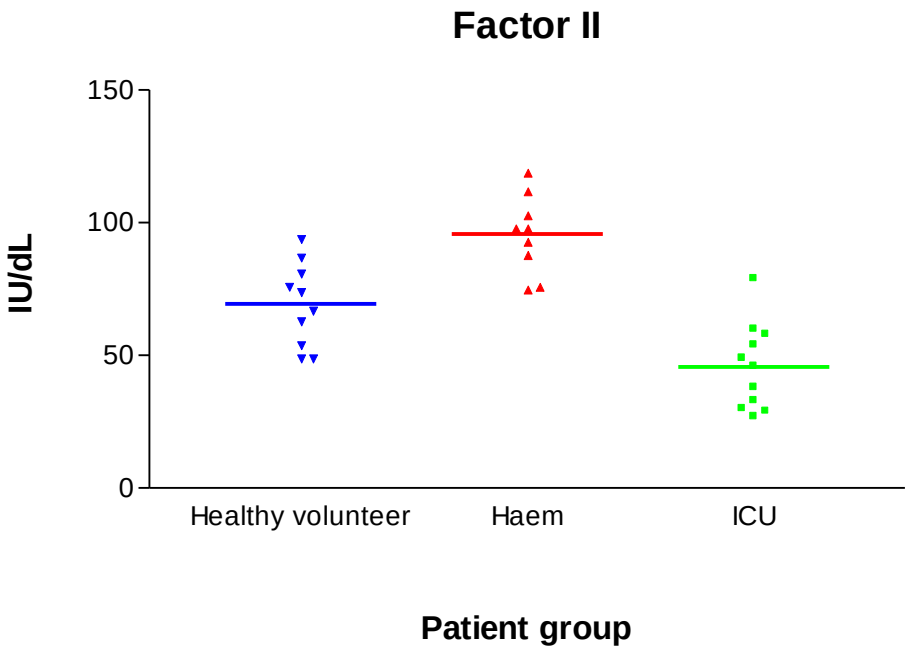


Figure 3.3a Clotting screens tested on platelet poor plasma n=10, Healthy volunteers; n=9, haemophiliac; n=11, ICU samples. (Haem-haemophilia group). Data points in each group are expressed as the mean of duplicate runs and the bars represent the mean of all the data points plotted within each sample group



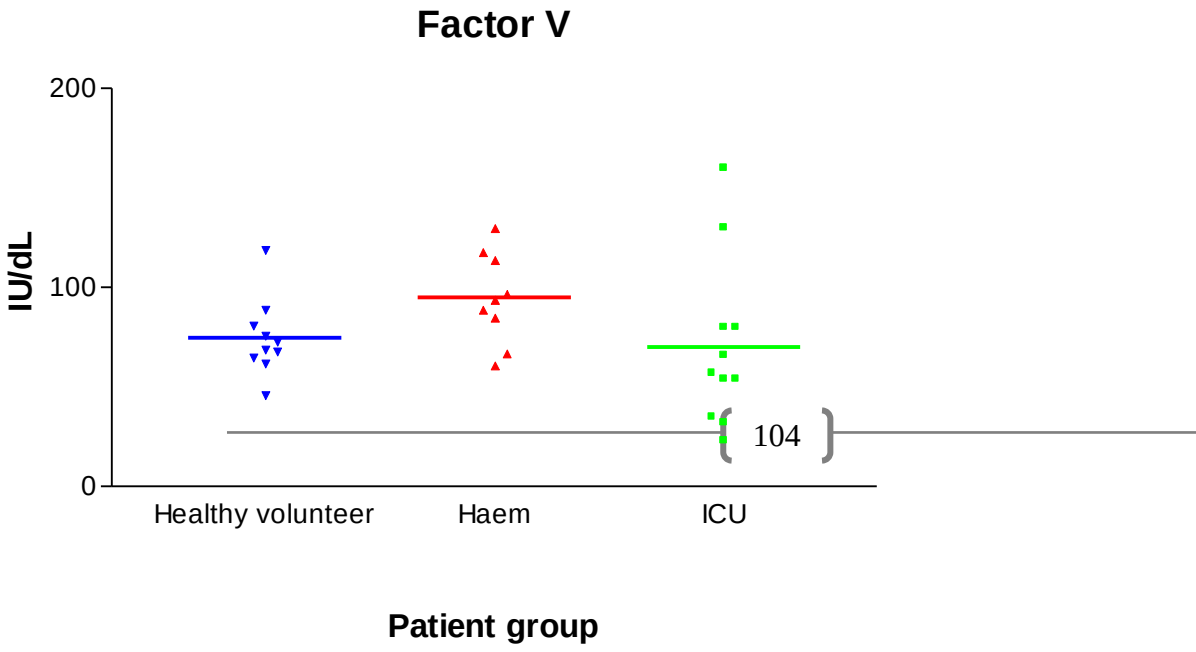
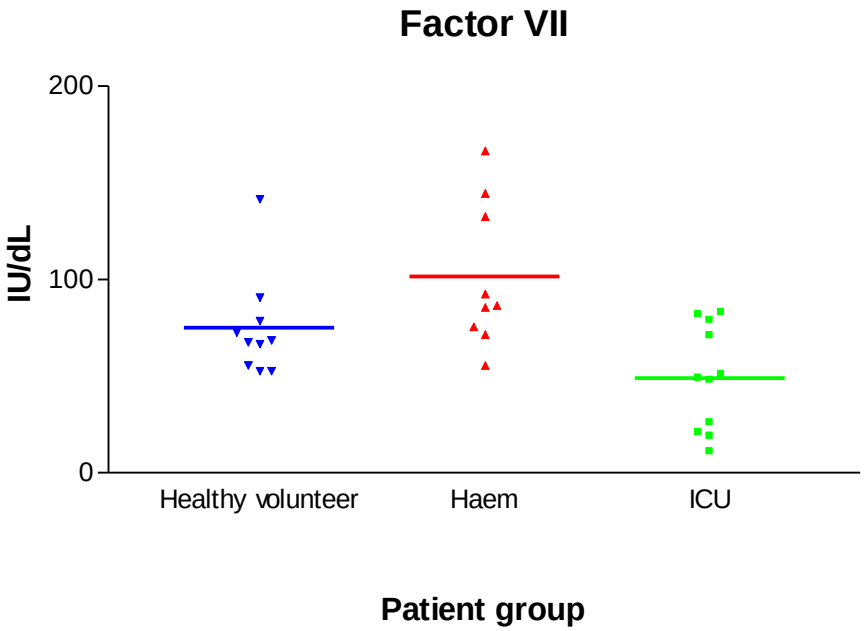


Figure 3.3b Prothrombin and FV levels tested on platelet poor plasma n=10, healthy volunteers; n=9, haemophiliac; n=11, ICU samples (Haem-haemophilila group). FII, factor II; FV, Factor V. Data points in each group are expressed as the mean of duplicate runs and the bars represent the mean of all the data points plotted within each sample group



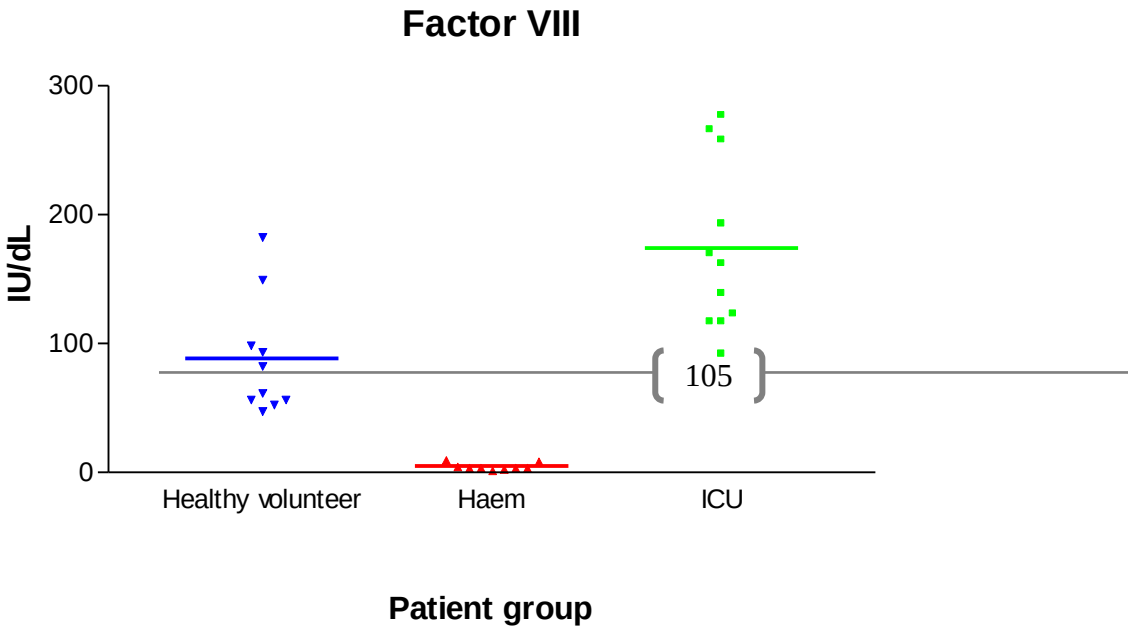


Figure 3.3c Factor FVII and FVIII levels tested on platelet poor plasma n=10, healthyvolunteers; n=9, haemophiliac; n=11, ICU samples (Haem-haemophilila group) FVII, factor VII; FVIII, Factor VIII. Data points in each group are expressed as the mean of duplicate runs and the bars represent the mean of all the data points plotted within each sample group

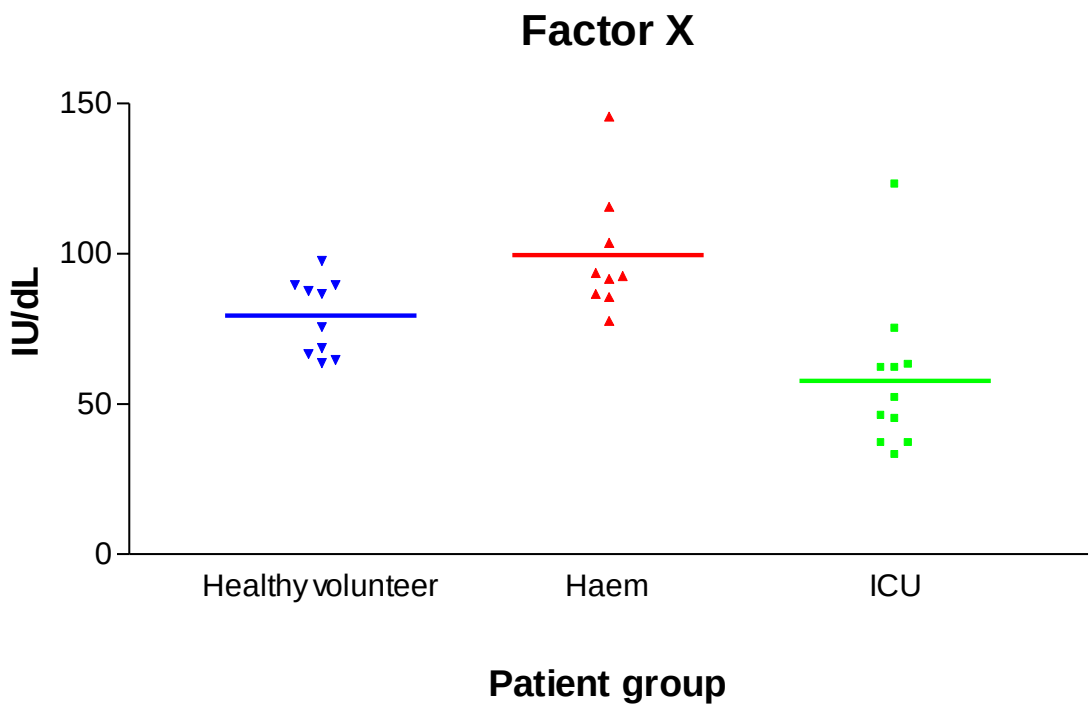


Figure 3.3d FX levels tested on platelet poor plasma n=10, healthy volunteers; n=9, haemophiliac; n=11, ICU samples (Haem-haemophilila group) FX, factor X. Data points in each group are expressed as the mean of duplicate runs and the bars represent the mean of all the data points plotted within each sample group

CONCLUSION: Within the ICU group, coagulation factors are significantly lower than the reference ranges (section 2.7). These results are consistent with the prolonged clotting screens observed. FVIII and fibrinogen levels were elevated; this could be consistent with an acute phase reaction in these patients. The raised DD (D-Dimer) levels will also have a significant effect in the stability of the clot formed. Comparatively, factor levels for the healthy volunteers and haemophilia groups were also assayed and are shown in Figures 3.3a-d.

3.2.2 Thrombin generation in the ICU sample group

The generation of FXa in haemophilia is reduced on the surface of the platelet due to the deficiency of in the Tenase complex. This results in insufficient thrombin generation, which ultimately leads to the reduction of thrombin-dependent clotting processes. This suggests that FVIII and FIX on the platelet surface have a regulatory role on the generation of thrombin.

The curve (Figure 1.3, blue trace) created during thrombin generation reflects the actions of the total haemostatic mechanism. The endogenous thrombin potential depicted as the area under the curve represents the pattern of thrombin formation. In this investigation, thrombin generation was measured in platelet poor plasma (PPP) samples collected from ICU patients and analysed. This analysis was carried out by a colleague who was analysing thrombin generation in this patient group.

Thrombin generation analysis was carried out as described in Section 2.7. The ICU plasma samples (80µl) were analysed and the reaction monitored using the thrombinoscope software. Five parameters were observed, namely *lag time* (T1), *ETP* (endogenous thrombin potential), area *under the curve* (T4), *peak of the curve* (T3), *time to the peak* (ttPeak) [T2] and the *start tail*.

Reference ranges for thrombin generation were established from plasma samples taken from 16 healthy subjects. These values are currently under further assessment as laboratory reference ranges. The reference ranges to date are shown (mean +/- 2SD) below in Table 3.3.

Variable	PPP n=16
Lagtime (min)	4.1-6.5
ETP (nmol.min)	114-2272
Peak (nmol)	92-287
ttPeak (mins)	8.5-12.5
Start tail (min)	25.1-35.3

Table 3.3 Thrombin generation references ranges and units. ETP, Endogenous thrombin potential; ttPeak, total time to peak

Analysis was carried out for the total thrombin generation potential within the ICU patient group (Table 3.4). Contact factor activation was eliminated as a pre-analytical variable using CTI. Of the 11 samples tested from the ICU group, one sample failed to initiate thrombin generation and therefore did not show any measurable thrombin production.

Sample	Lag time	ETP	Peak	ttPeak	Start tail
1	20.50	720.5	55.40	27.0	54.0
2	6.40	1290.0	113.50	11.1	41.5
3	6.30	1138.0	119.60	10.7	39.0
4	11.70	958.5	115.70	16.1	39.5
5	13.20	653.5	71.50	18.0	42.0
6	8.70	1727.0	138.10	13.7	51.0
7	21.70	597.0	37.40	31.2	57.0
8	***#*** **-**	***0.0***	**-**	**-**	**-**
9	7.00	1524.5	128.80	15.1	42.0
10	14.50	1358.5	-	119.24	19.13
11	6.75	1402.5	-	166.11	10.88

Table 3.4 Thrombin generation evaluation in ICU (samples n=11). [#] One of the samples did not show any thrombin generation capacity (bold and italics) ETP, Endogenous thrombin potential; ttPeak, total time to peak

In relation to the reference ranges, initiation of thrombin generation (11.68 ± 1.827), the ttpeak (42.83 ± 17.15) and the start tail (39.60 ± 4.594) were markedly reduced in ICU samples The average ETP (1034 ± 152.2) in these samples was also reduced which could indicate a reduced rate of thrombin generation. The time taken for the thrombin concentration to return to baseline was markedly prolonged.

CONCLUSION: The thrombin generation in the ICU sample group demonstrated a prolonged initiation stage and the total thrombin generated in this sample group was reduced. The prolonged start tail observed could suggest a diminished stability of the fibrin clot formed.

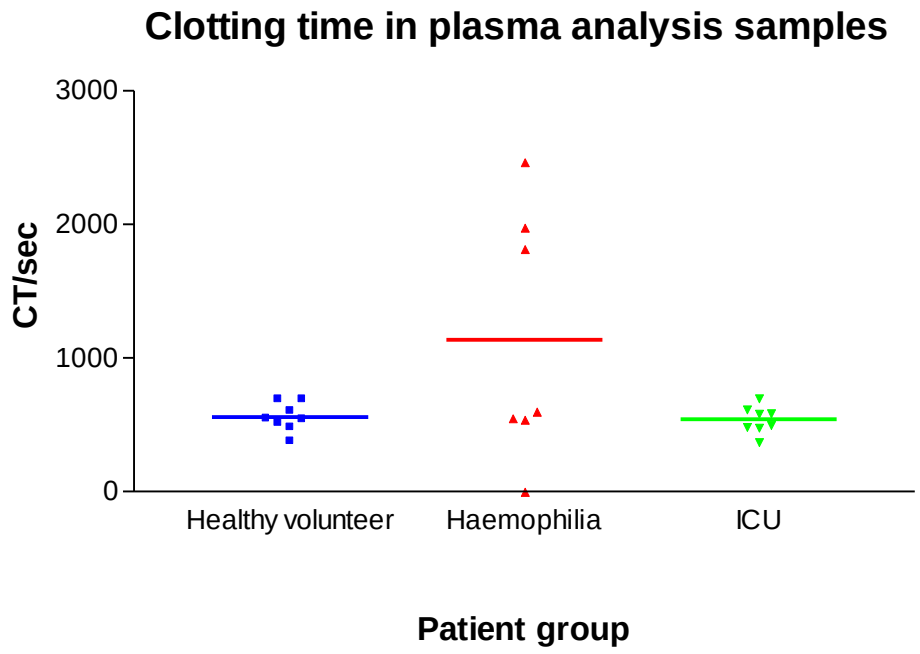
3.3 INVESTIGATION OF CLOTTING PROFILE WITHIN A PLASMA SYSTEM

3.3.1 Profile of fibrin clot formation in a plasma system

Following the preliminary analysis of whole blood profiles (Section 3.1), investigation of fibrin formation in a plasma system was analysed. Previous investigations have shown that addition of rFVIIa improved both the time and rate of clot formation (Gabriel *et al*, 2004; Sorensen and Ingerslev, 2004; Collins *et al*, 2006).

In Sections 3.1 – 3.2, the results demonstrated that haemostasis in ICU patient samples was altered compared to samples from the healthy volunteer group. Furthermore, the addition of rFVIIa normalised or enhanced the production of thrombin and clot formation. The aim of analysing fibrin clot formation in a plasma system was to assess the *in vitro* clotting profile in these samples, in a non-cellular system looking only at the interaction of plasma components.

Samples were analysed using the ROTEM assay as explained in Section 2.6 and only altered when the infusion of 5µl of the rFVIIa, at variable concentrations, (see Appendix ii) was added to the sample. Results are shown in Figures 3.4 – 3.7.



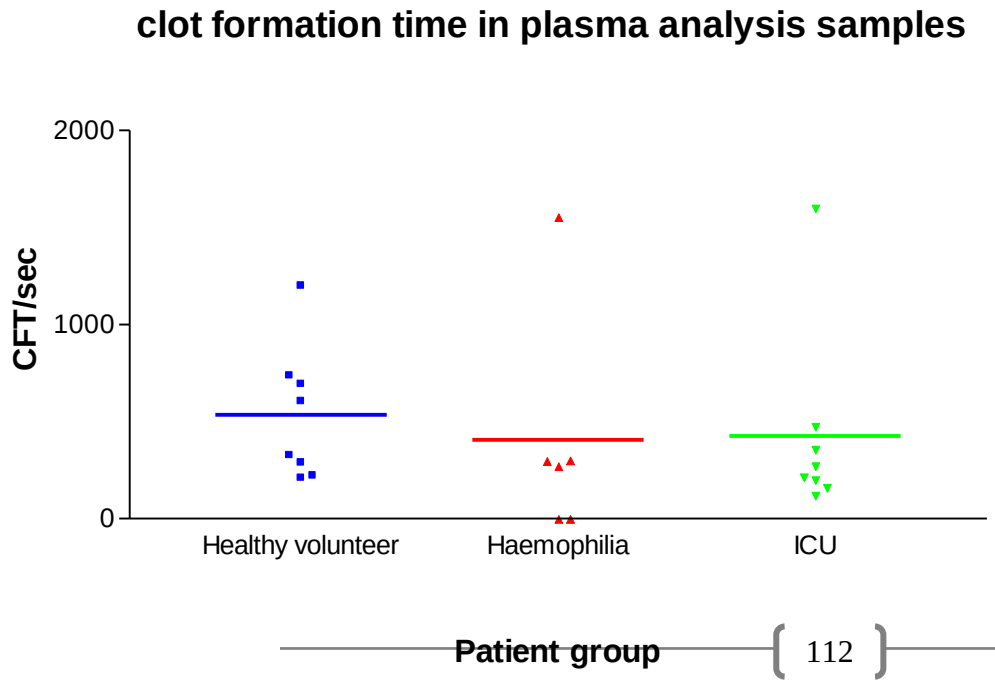
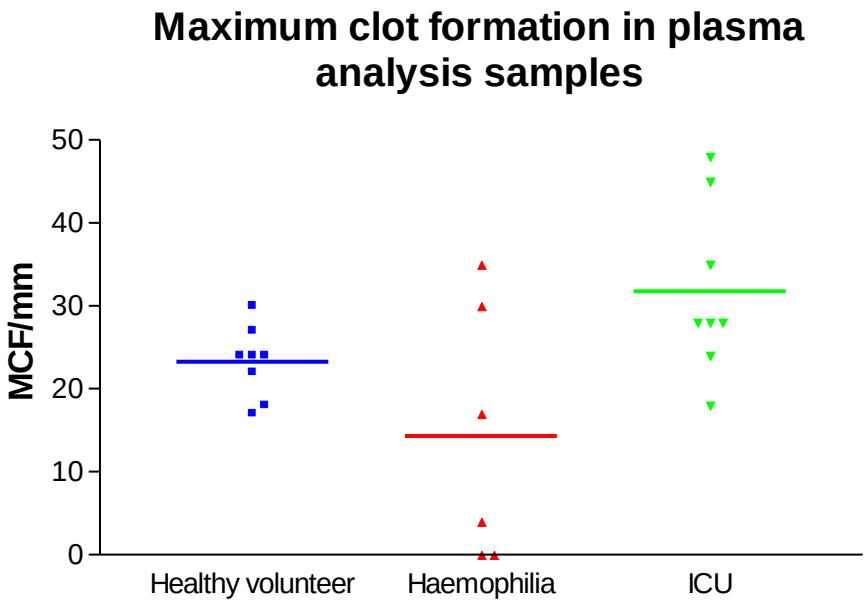


Figure 3.4a Clotting times (seconds) and the rate of clot formation (CFT) recorded during plasma analysis of the with the ROTEM assay. Graph shows healthy volunteers (*blue*, n=10), Haemophiliac (*red*, n=9) and ICU (*green*, n=11) samples. CT, clotting time. Data points in each group are expressed as the mean of triplicate runs and the bars on each graph represent the mean of all the data points plotted within each sample group



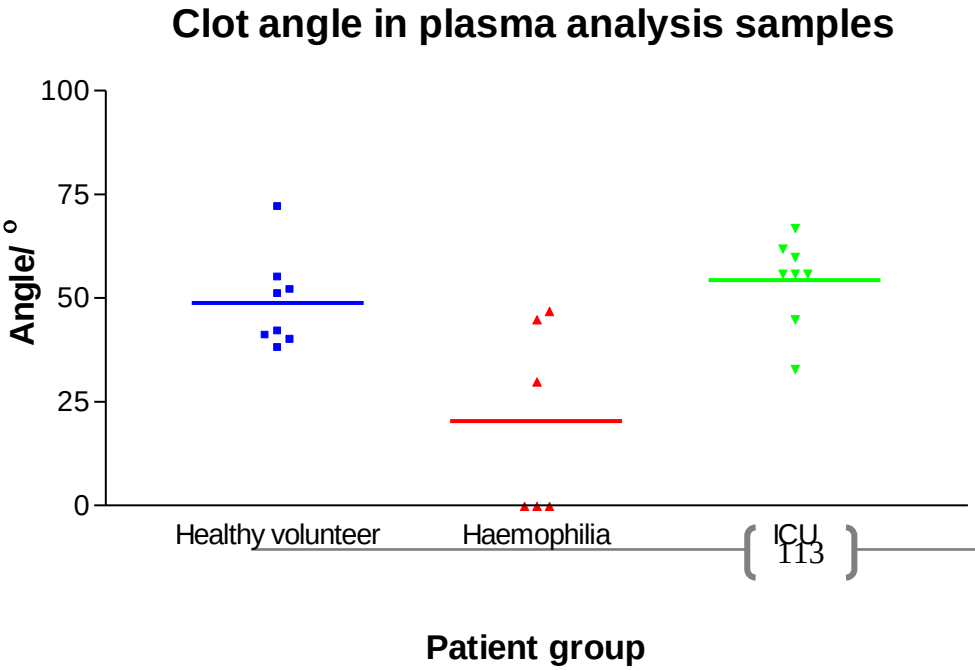


Figure 3.4b Maximum clot formation (MCF) and clot angle recorded during plasma analysis of the with the ROTEM assay. Graph shows healthy volunteers (*blue*, n=10), Haemophiliac (*red*, n=9) and ICU (*green*, n=11) samples. Data points in each group are expressed as the mean of triplicate runs and the bars on each graph represent the mean of all the data points plotted within each sample group

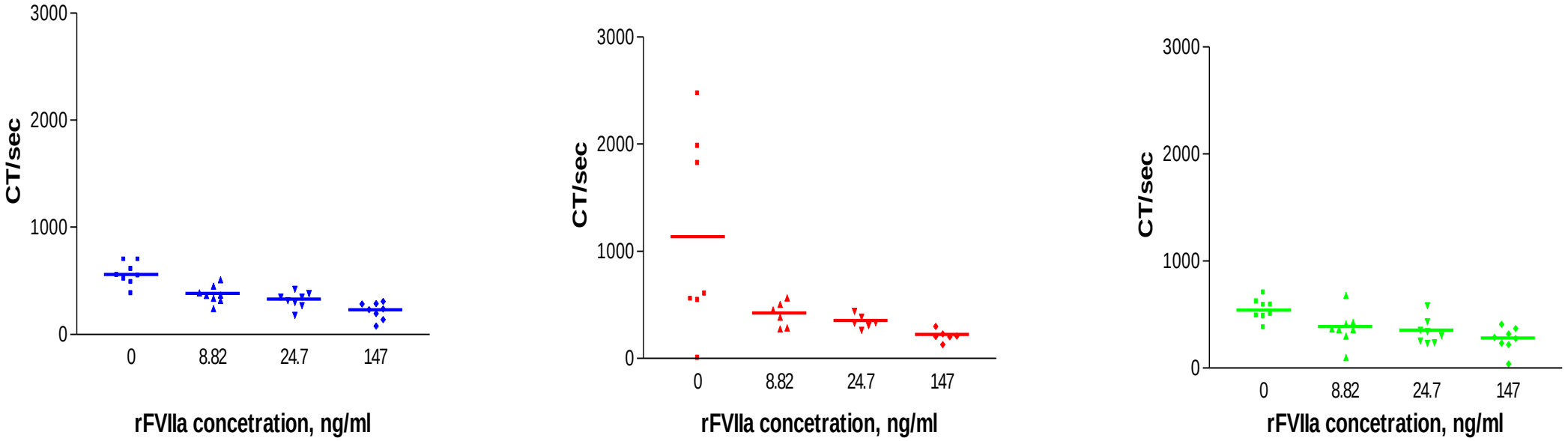


Figure 3.5 Clotting times (seconds) recorded during plasma analysis with the ROTEM assay in healthy volunteers (*blue*, n=10), Haemophiliac (*red*, n=9) and ICU (*green*, n=11) samples. CT, clotting time; rFVIIa, recombinant activated FVII. Data points in each group are expressed as the mean of triplicate runs and the bars on each graph represent the mean of all the data points plotted within each sample group

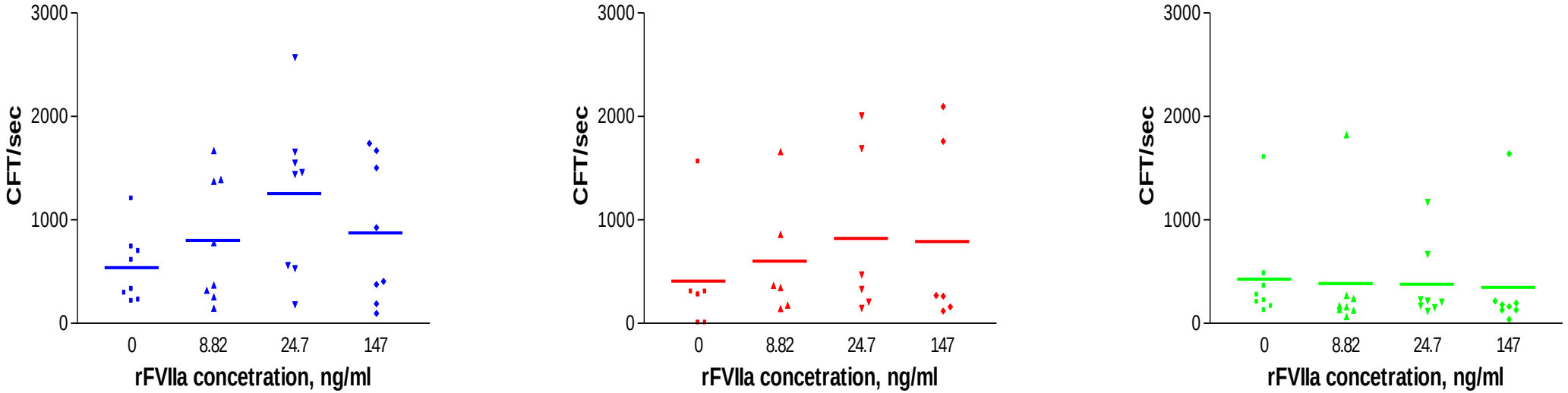


Figure 3.6 Rate of clot formation recorded as the clot formation time (seconds) recorded during plasma analysis with the ROTEM assay in healthy volunteers (blue, n=10), Haemophiliac (red, n=9)) and ICU (green, n=11) samples. CFT, clot formation time; rFVIIa, recombinant activated FVII. Data points in each group are expressed as the mean of triplicate runs and the bars on each graph represent the mean of all the data points plotted within each sample group

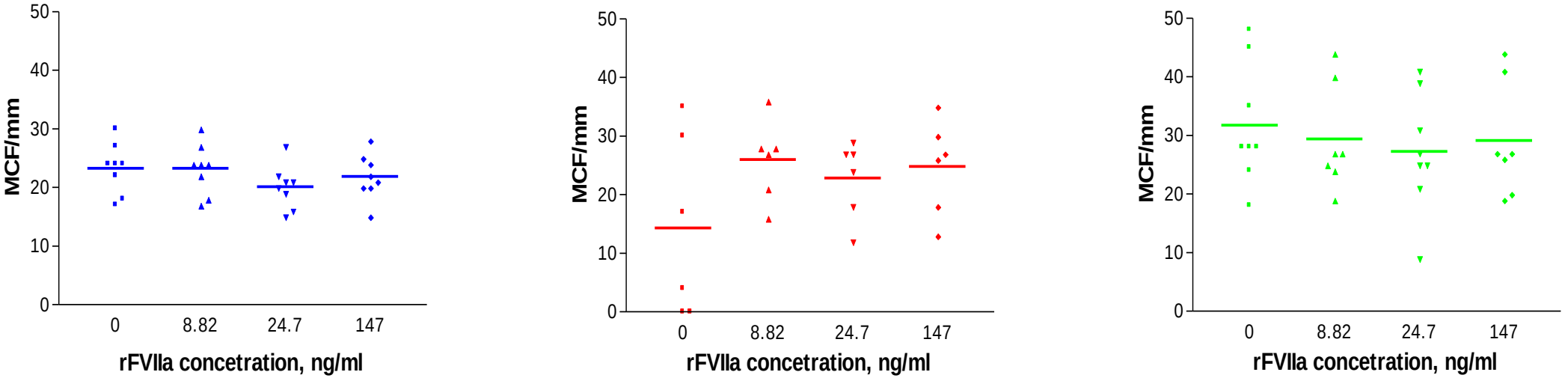


Figure 3.7 Maximum clot firmness of the clot (mm) recorded during plasma analysis with the ROTEM assay in healthy volunteers (blue, n=10), Haemophiliac (red, n=9)) and ICU (green, n=11) samples. MCF, maximum clot formation; rFVIIa, recombinant activated FVII. Data points in each group are expressed as the mean of triplicate runs and the bars on each graph represent the mean of all the data points plotted within each sample group

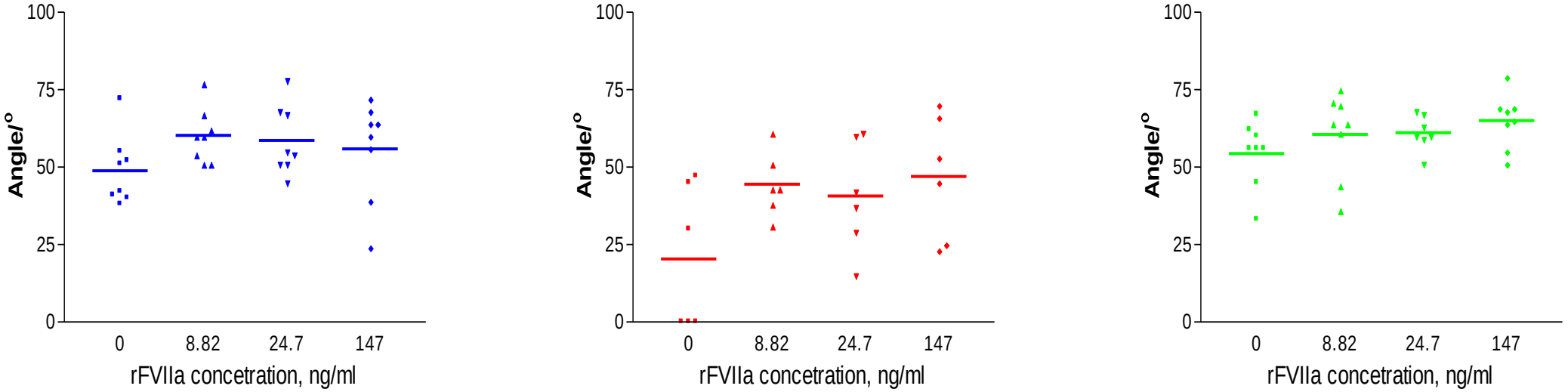


Figure 3.8 Clot angle (degrees) recorded during plasma analysis with the ROTEM assay in healthy volunteers (blue, n=10), Haemophiliac (red, n=9)) and ICU (green, n=11) samples. rFVIIa, recombinant activated FVII. Data points in each group are expressed as the mean of triplicate runs and the bars on each graph represent the mean of all the data points plotted within each sample group

Intra-variation within each group

Figure 3.4 describes the clot formation parameters of the ROTEM within each sample group. The start of fibrin clot formation and the rate at which this fibrin clot forms is shown in figure 3.4a. Haemophilia sample group have a significantly higher clotting time than the healthy volunteer and ICU sample group, whose clotting times are similar. According to this data, the time taken to clot formation is the same in the healthy volunteer and the ICU sample group. However, once the fibrin clot is initiated, the rate at which more fibrin formation occurs is similar in the ICU and haemophiliac group. Figure 3.4b represents the size of the fibrin clot formed. The mass of the clot formed (MCF) is higher in the ICU sample group and the healthy volunteers than in the haemophilia group.

Responses to rFVIIa within each sample group showed significant reduction in clotting time in response to all three concentrations of rFVIIa (normal = $p<0.0001$; Haemophiliac = $p<0.01$; ICU = $p<0.002$, ANOVA) as shown in Figure 3.5. However, there was no statistically significant variation between the rFVIIa concentrations in the CFT, MCF and the clot angle within each of the patient groups.

Inter-variation among the groups

Clotting times: The haemophiliac and ICU samples demonstrated longer clotting times in comparison to samples from healthy volunteers. The ranges of the clotting times were more noticeable within the haemophiliac group although this variation is not statistically significant ($p>0.05$ ANOVA). Following the addition of rFVIIa, the

clotting times were reduced especially within the haemophiliac sample group. All three groups showed that clotting times are responsive to rFVIIa infusion.

Rate of clot formation: The clot formation times were longer in the haemophiliac and ICU sample groups. Addition of rFVIIa increased the rate at which clots were formed on the ROTEM assay. The fastest rate of clot formation was observed in the healthy volunteer sample group, followed by the haemophiliac group. ICU samples showed little improvement in the rate of clot formation following the addition of rFVIIa to the plasma sample. The rate of clot formation showed no statistical significance overall ($p>0.05$ ANOVA).

MCF and the angle of the clot: ICU samples demonstrated the highest MA of the clot as well as the angle of the clot. The haemophiliac samples showed a varied but lowered MCF and clot angle size. Following the addition of rFVIIa, there was a significant increase in both the MCF and clot angle ($p<0.02$ and $p<0.01$ respectively). There was no statistical significance in MCF and clot angle with increased rFVIIa infusion into the sample using ROTEM analysis in all three sample groups. This may be indicative of the role played by rFVIIa at the initiation of fibrin clot formation rather its progression, especially in the absence of cellular components.

3.4 ANALYSIS OF THE FIBRIN CLOT LYSIS PROFILE WITHIN A PLASMA SYSTEM

3.4.1 Fibrin clot lysis

In addition to the ROTEM, coagulation and thrombin generation tests, clot lysis assays were devised so as to analyse the stability of the plasma clot in ICU samples.

An assay that employs both coagulation and fibrinolysis was used to assess the fibrinolytic potential in these samples. Fibrinolysis plays a significant role in various haemorrhagic and prothrombotic conditions, just like the excessive fibrinolysis seen in severe haemophilia A, liver damage and the defective fibrinolysis observed in renal failure (Mosnier *et al*, 2001, Colluci *et al*, 2003, Lottermoser *et al*, 2001).

This new global assay was used to simultaneously measure coagulation and fibrinolysis i.e. the clot formation and lysis assay (CLoFAL) assay. This method is based on the procedure used by Lisman et al, 2002 and Goldenberg et al, 2005. Reagent concentrations were optimised for this assay as described in Section 3.6. Platelet poor plasma samples were thawed at 37˚C and placed on ice until the time of assay (for less than 25 minutes incubation on ice). Reaction lysis buffer was prepared from tpa (3μg/ml), phospholipid (100μM), $CaCl_2$ (0.2M) and tissue factor (9.3ρM) to give the following final concentrations (tpa; 375ng/ml, PL; 12.5μM, CaCl2; 2.5mM and TF; 1.1625ρM) after addition of the reactant solution to plasma samples.

All measurements and calculation were derived from a curve generated after an initial baseline absorbance at time T_0 followed by a progressive rise in absorbance until maximum peak (MP) was reached at time T_1. This was followed by a phase of decline

in absorbance after which a late phase of decline to baseline in absorbance was noted at time T_2. This is illustrated in Figure 3.8b.

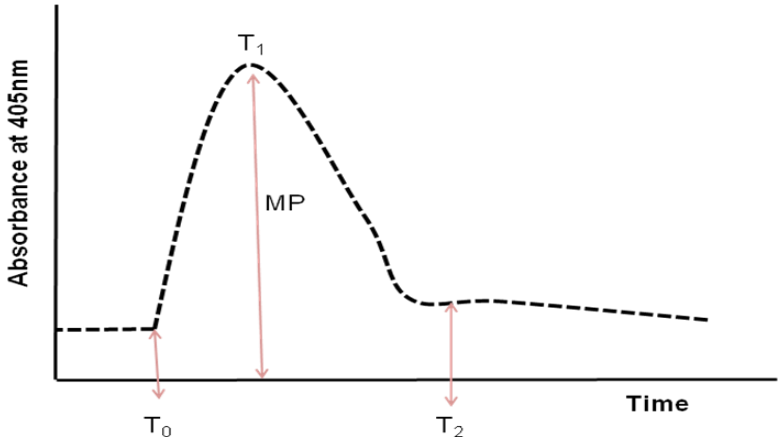


Figure 3.8b Representation of the CLoFAL curve derived from standard normal pooled plasma, illustrating the parameters of the CLoFAL principle

The clot lysis assay method has been adapted by generating a fibrin clot produced from platelet-poor plasma in a microtitre plate system and measuring the dissolution of these clots using an automated approach. This is an extension of the previous analyses carried on the individual patient group samples. This method requires an extended time period and sample incubation at 37°C. Samples were analysed using the CLoFAL assay as discussed in Section 2.6. The Typical curves for healthy volunteer, haemophilia and ICU sample groups generated, using platelet poor plasma, are shown in Figure 3.9.

3.4.2 Clot stability

Figure 3.9 shows analysis of clot formation and lysis in platelet poor plasma from each respective patient group in the absence of rFVIIa. The plasma samples were added to

a 96 well plate, in the presence of tpa to induce lysis, which was observed and recorded over 150 minutes. The absorbance measured and recorded was calculated as the amount of light restricted through the sample as the clot formed and inversely as the sample lysed.

Healthy volunteer plasma showed a gradual rise in clot formation. The same pattern during the initiation phase of clot formation was noted in the ICU samples although active formation of the clot happened 5 minutes after the formation recorded in the healthy volunteer plasma samples. The haemophiliac samples showed much lower maximum amplitude than the other sample groups. The ICU and haemophilia samples did not show a decline in absorbance. The change in the peak absorbance units (healthy volunteer 118.4, haemophilia A 86.66 and ICU 130.3) among the three groups is statistically significant ($p<0.0001$).

CONCLUSION: The variation in responses within each group could be an indication of the type of clot formed in the presence of altered coagulation components.

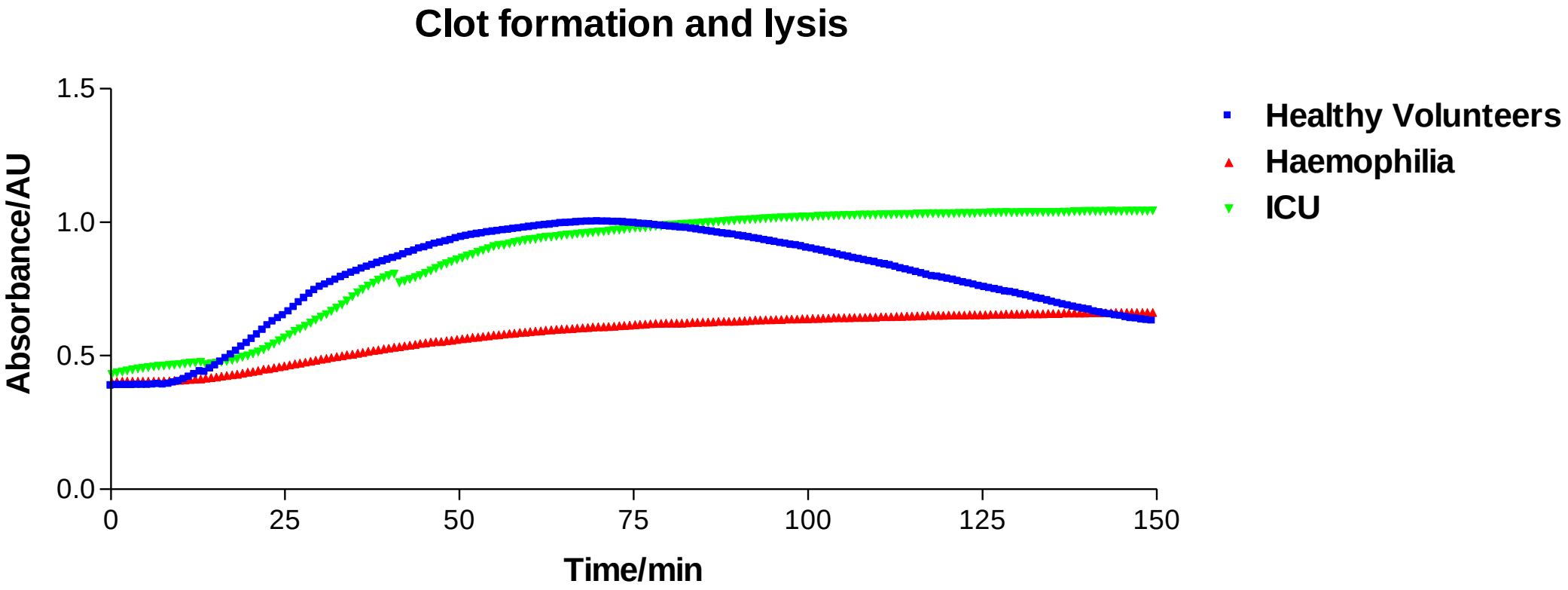


Figure 3.9 Clot formation and lysis in the absence of rFVIIa using the CLoFAL assay in all three sample groups. Healthy volunteers (blue, n=10), Haemophiliac (red, n=9)) and ICU (green, n=11) samples. Data points in each group are expressed as the mean of triplicate runs

CHAPTER 4:

DISCUSSION

Patients in the intensive care unit (ICU) often present with a variety of pathophysiologic conditions that are associated with abnormal haemostasis. Bleeding is a major risk in patients with inherited or acquired coagulopathies. Those experiencing massive trauma or undergoing major surgery also present a special challenge to the ICU. Bleeding complications usually result from abnormalities in platelets or deficiency of coagulation factors, reduced thrombin potential, and altered fibrinolytic pathway and require specific blood or coagulation factor replacement. The development and advancement of diagnostic techniques and the availability of new potent therapeutic agents have improved the care for these patients.

The data presented in the present studies has demonstrated that samples taken from ICU patients have abnormal coagulation. These ICU samples had abnormal clotting screens with delayed initiation of haemostasis, which was measured by three low TF-activated global haemostasis assays, namely ROTEM, CAT and the CLoFAL assay systems. Clotting profiles in whole blood indicated a variation to that seen in plasma analysis. However, one common observation among between whole blood clotting and plasma clotting was that once haemostasis was activated, the propagation and formation of the clot was either normal in some ICU samples or enhanced. This is explained further on.

Currently, it has been proven that low tissue factor can activate haemostasis *in vitro* (Sorensen *et al*, 2004). The rate at which the clot forms, particularly, the rate of thrombin production, is vital for stable fibrin clot formation (Mann *et al*, 2003 and Roberts *et al*, 2004). Routine clotting screens are often prolonged in critically ill patients as demonstrated in this study. However, these tests fail to reflect the haemostatic status of the patient. It could be that the PT and APTT are depicting a

specific stage during the initiation phase in haemostasis where by the start of clot formation has been delayed by various factors such as the presence of increased inhibitors or contact factors. Clinically, therapeutic intervention for prolonged clotting screens is FFP infusion, which based on the previous argument, may not be necessary unless the patient's factor levels are significantly reduced. The three global assays used in this study observed if there was a delay during the initiation of clot formation and it was demonstrated that there was a similarity in pattern of results demonstrated in the clotting screens and the global assays. The CAT assay results showed that the initiation of thrombin generation in the ICU samples was delayed and the peak thrombin reduced, however the total amount of thrombin measured by the ETP was similar to the reference ranges derived from healthy subjects. Similarly, the time to clot formation in both the ROTEM and the CLoFAL assay was also delayed but the size of the clots and rate at which they were being formed was comparable to the healthy volunteer group and slightly enhanced in some of the ICU samples.

The prolonged clotting screen and clotting times in conjunction with the slightly enhanced clot size recorded from these studies may be explained by the individual factor levels measured based on the current accepted models of haemostasis. TF/FVII pathway activates coagulation, which leads to a small amount of thrombin being generated. This thrombin is not enough to allow fibrinogen activation but is sufficient for FV and FVIII activation and the expression of negatively charged proteins on platelet surface. The tenase complex of FIXa and FVIIIa, along with the prothrombinase complex of FXa and FVa localised on the platelet surface, drives the large thrombin burst required to form a stable clot (Mann *et al*, 2003 and Roberts *et al*,

2004). Factors FII, FV, FVII and FX were decreased in the ICU sample group. FVII is required during the initiation stage of thrombin production to activate FX to FXa so that the initial small amount of thrombin is generated. This small amount of thrombin then activates FVIII and more platelets to bring about a much a larger thrombin burst. A decrease in FVII and also FX may be consistent with insufficient thrombin production during the initiation step of coagulation. These factors are required to activate factors V and VIII and to stimulate platelet expression of phospholipids. Alternatively, FVIII levels were either normal or increased in ICU samples.

4.1 Standard laboratory screening tests

The prolonged PT and APTT tests and the delayed initiation of coagulation demonstrated by the global assays may communicate indistinguishable results in terms of the initiation of clot formation. Speculatively, this may limit the effectiveness of the initiation time of the global assays and so other parameters that were measured and recorded may be of more use. Even though collectively the ICU sample group in this study showed a somewhat prothrombotic state (Figure 3.2A, Table 3.4 and Figure 3.4b), singularly most samples showed parameters that were below the reference range (CFT and the MCF), which suggests an abnormality in clot formation.

4.2 Whole blood analysis

Thrombocytopaenia is a major complicating factor in the ICU setting. In order to asses clotting in whole blood, a platelet count was initially conducted on each sample taken from ICU patients as well as the comparative control groups (haemophilia and healthy volunteer groups). The main clinical significance of measuring the platelet count is that

it is a good indicator for increased bleeding risk. This analysis was carried out first to ascertain what the platelet counts were in these samples and whether platelets influenced whole blood clotting profile when assayed using the ROTEM.

Figure 3.1 illustrates the findings from the platelet counts carried out on all three patient groups. The ICU samples demonstrated decreased platelet counts in comparison to the haemophilia and healthy volunteer group. ICU samples also showed the widest range of platelet counts from $12.20x10^9$/L to $451.1x10^9$/L.

Whole blood analysis carried out on the ROTEM assay recorded four parameters. The clot angle and maximum clot firmness (MCF) represents the size of the clot formed, which in whole blood analysis is influenced by the presence of platelets and other cellular components. Figure 3.2A showed that MCF in the healthy volunteer group was similar to the ICU sample group but the haemophilia sample group showed the lowest MCF and clot angle. thrombocytopaenia, as discussed in section 1.5.1, is a result of various factors. The measurement of the platelet count is a specific single assay that is focussed on one aspect of haemostasis in these ICU samples. It is represented as a single entity within a system where components of both primary and secondary haemostasis are being affected in various ways. The ROTEM assay results shown in Figure 3.2A demonstrate that the clotting in whole blood from the ICU samples is comparable to the results seen in the healthy volunteer group. This is inconsistent with the platelet count results. However, it should be considered that without further clinical information on these patients, it is appropriate to wholly compare the clotting profile in these two study groups. If there are predisposing factors such as sepsis, DIC or microangiopathy in the ICU group then it is likely that these are contributing to the

comparable MCF and clot angle in ICU sample group, especially if the DIC-contributing factors are favouring a hypercoagulable state. During sepsis, the presence of haemophagocytosis may occur resulting in platelet clearance. However the increase in inflammatory response will lead to increased thrombin production, which will subsequently favour a thrombogenic profile, as observed in microangiopathic patients. Therefore, even though platelet counts are low in the ICU sample group, the TEG data shows that this is not reflected in the ROTEM traces. Speculatively, the variation in the plasma pro- and anti-coagulation proteins may play a significant role in this as discussed earlier.

TF/FVIIa interaction is necessary for the initiation of haemostasis. rFVIIa binds TF in the same manner that FVII binds TF during initiation. rFVIIa has been shown to directly activate FX on the surface of locally activated platelets *in vivo*, which initiates the thrombin burst independently of FVII and FIX as well as TF. The addition of rFVIIa to whole blood samples illustrated a haemostatic response as shown in Table 3.2. The ICU sample group showed the least response to the addition of rFVIIa in comparison to the healthy volunteer group and the haemophilia group. The CT represents the time it takes for the clot to start forming and the CFT represents the rate at which the clot is formed once the formation is initialised. The time to CT in the ICU sample group was increased by 33% and CFT by 57% in the presence of rFVIIa but the MCF was not increased. Addition of rFVIIa to these whole blood samples did not increase the MCF of the clots and since it had already been demonstrated that there were low platelet counts in this sample group, the platelet count could be one of the factors influencing the MCF. Minute concentrations of thrombin produced during the initiation stage of

coagulation are sufficient for platelet, FV and FX activation *in vivo* but rFVIIa can mediate a large thrombin burst in the presence of phosphatidylserine, which is abundant in the cell membrane of platelets. Therefore it would seem that the subsequent reactions during clot formation are influenced by both the cellular and plasma protein content. If there is a deficiency of platelets and plasma factors initially then optimal thrombin and fibrin production will not be efficiently maximised, even in the presence of rFVIIa. The highest response to rFVIIa was noted in the haemophiliac sample group. The lack of FVIII in the haemophiliac sample group results in inefficient activation of FX (Section 1.7.2). According to the cell-based model of haemostasis, it isn't that the TF-generated FX is insufficient but rather that it occurs on the wrong cell surface. Normally, the FIXa/FVIIIa complex generated activates FX on the platelet surface during propagation and the TF/FVIIa activates FX on the TF-bearing cell. FXa produced on the TF-bearing cell cannot move to the activated platelets because it is liable to inhibition by plasma FXa inhibitors, antithrombin and TFPI. Normally during circulation, both TFPI and AT inhibit FXa rapidly and effectively, with a half-life of 1 minute or less for FXa in the fluid phase. FXa that remains at the TF-bearing cell is protected from inhibition, whereas any FXa that diffuses from this cell surface is quickly inhibited. This indicates that haemophilia is a result of failure of platelet-surface activation of FX. The initiation and amplification stages of coagulation are relatively normal in haemophilia suggesting that the initial platelet plug can be formed but the thrombin burst necessary to stabilise this initial plug at the platelet surface cannot be generated. Previous studies have shown that rFVIIa directly activates FX on the surface of locally activated platelets. This activation initiates the thrombin burst" independently of FVIII or/and TF. Plasma factor levels in ICU samples are discussed further in Section 4.3.

4.3 Fibrin clot formation

In order to analyse clot stability using a plasma system, another assay was used. The modified CLoFAL assay derived from the methods published by Lisman et al, 2002 and Goldenberg et al, 2005 employs both clot formation and clot lysis. Because this assay quantitatively measures the rate at which the clot is formed and lysed, it was used to analyse the rate at which the formed fibrin clots within a plasma system were being cleared in the presence of a standardised concentration of tpa. This analysis is highly influenced by the biochemical composition of the plasma.

Like the ROTEM assay, the CLoFAL assay also uses low TF to initiate clot formation and low tpa concentrations to initiate fibrinolysis. The influence of coagulation factor deficiencies has previously been investigated (Goldenberg *et al*, 2005). Fibrinogen and FVIII have been reported to influence the MP and T1 and hence the rate of clot lysis. ICU samples showed a prolonged time to the maximum peak, which is comparable to the CFT and MCF of the ROTEM assay. In Figure 3.9, the CLoFAL analysis demonstrated a decreased response in the haemophiliac sample group but the ICU sample group demonstrated a similar pattern to the healthy volunteer group. This is similar to what was observed in the clotting profile recorded with the ROTEM in that the MCF and the angle of the clot was enhanced. The CLoFAL assay is a turbidimteric assay and therefore the rise and decline in absorbance corresponds to clot formation and lysis but more specifically fibrin polymerisation and reorganisation.

4.4 Fibrin architecture

Recent studies have shown hat abnormal thrombin generation produces clots that have altered fibrin structure, changes that are associated with bleeding or thrombotic risk. Several factors influence clot formation (such as the presence of cells and their products such as soluble factors like TAFI and PAI-1) but the most important is the thrombin present during fibrin clot formation. Very low concentrations of thrombin are sufficient to facilitate fibrinopepetide cleavages, resulting in fibrin polymerisation. Low thrombin concentrations produce fibrin clots that are turbid and composed of thick, loosely-woven fibrin strands whereas higher fibrin concentrations produce thinner, more tightly packed fibrin strands (Wolberg, 2007).

Fibrin plays two roles in fibrinolysis, it acts a both a substrate and a co-factor for plasmin, and may explain why clots with altered fibrin structure exhibit altered susceptibility to fibrinolysis. Thinner fibrin fibres show a slower rate of tpa-mediated lysis, thereby reducing the rate of overall fibrinolysis in comparison to thicker fibres (Gabriel et al, 1992). Consequently, clots with thinner fibres are more resistant to fibrinolysis, hence thicker fibrin fibres lyse slower than thin fibres. However, because clots that are produced by low thrombin concentration are composed of thick fibres that are loosely woven, there will be less fibres in the clot, which makes the clot more susceptible to lysis. High thrombin concentration clots have more tightly packed fibres with more individual strands. Collectively, this means that even though plasmin is generated on individual fibrin fibres, its activity is rate-limited within the entre fibrin clot structure.

The way thrombin generates a clot has further been explained through the extrinsic and intrinsic activities (Wolberg, *et al*, 2005). It is believed that low thrombin levels are produced during the extrinsic pathway and further on during coagulation, at the propagation step, higher concentration of thrombin are produced. This means that the fibrin architecture is composed of a mixture of both thick and thin fibrin fibres. This can be clearly explained in a haemophilia setting. Deficiency of FVII or FIX causes a defective tenase complex. The results in a defective propagation stage, which means that the clot formed is composed of the thick fibrin strands produced during the extrinsic-initiation production of low concentration thrombin. Thick fibrin strands are more susceptible to fibrinolysis.

Results of the CAT assay showed reduced thrombin generation in ICU samples. Reduced thrombin levels decrease the rate of fibrinopepetide release and therefore delay fibrin polymerisation. Abnormal fibrin structure in the samples could render these clots susceptible to fibrinolysis. The reduced thrombin generation may also limit the activation of TAFI in plasma, which further allows fibrinolysis.

Recombinant activated FVII at high concentrations increases thrombin generation by directly activating FX. This shortens the time taken to reach peak thrombin level. ICU sample fibrin clots did not show an indication of lysis as was noted in the plasma fibrin clots from the healthy volunteers. This is suggestive of a less porous, lysis- susceptible clot structure. This may be because the fibrin strands are thinner and more compact with the overall clot structure. The robustness of the clot may be increased by the presence of rFVIIa. The high thrombi concentration may also influence TAFI activity, thus reducing the rate at which the clot lyses in an *in vitro* setting.

The role of clot structure in the ICU sample is important in that depending on when fibrinolysis is initiated, it could be a good indicator for bleeding or thrombosis. At the end of clot formation, both thick and thin fibres are present in the architectural structure. If clot lysis were begin after the clot is formed then the structure is likely to play a significant role in the stability of the clot formed and whether an anticoagulant or a procoagulant state is the likely outcome. Alternatively, if fibrinolysis occurs before the clot is fully formed, then it is more likely to be the competitive rate of clot formation and clot lysis that determines how haemostasis occurs. How which of these possibilities will occurs may depend on the clinical setting of the ICU individual and any other contributing factors to their clinical picture. Therefore, the pattern of thrombin generation may determine the clot structure formed and therefore determine the rate of clot lysis although this still requires extensive studies in the setting of these research aims.

CONCLUSION

The aim of this study was to analyse the clotting status in ICU patient samples through use of global assay analysis. This study has demonstrated that samples taken from ICU patients have abnormal clot formation and are susceptible to lysis, even in the presence of rFVIIa compared to their healthy volunteer counterparts. The global assays were used in conjunction with other laboratory assays to profile clot formation and stability in the ICU samples. Global assays activated by low TF showed that these ICU samples had a delayed activation of haemostasis but once initiated, thrombin and clot formation is comparable to that in healthy volunteers or somewhat enhanced. The addition of rFVIIa only improved the initiation of clot formation but overall did not significantly improve the clotting profile in the ICU samples but this, however, does not mean that rFVIIa is ineffective in ICU samples. Coagulation tests have been previously reported to show abnormal results in critically-ill patients (Collins *et al*, 2006) in the ICU and this has been shown in this study.

Previous reports have shown that abnormal haemostasis is a common occurrence and that it not insignificant. Haemostasis abnormalities contribute to mortality and morbidity and require thorough analysis to establish the underlying cause and to ensure that the correct intervention is carried out. Routine coagulation tests are used, mostly prior to surgery and other invasive procedures. This is usually based on the assumption that these first-line tests will clinically predict the risk of bleeding. However, first-line coagulation tests have limitations; especially clarification within a clinical setting. Routine coagulation tests may not be useful in a surgical and medical setting because they have limited sensitivity and specificity (Chee and Greaves, 2003) and this has been demonstrated in this study. If analysis of haemostasis was based on the clotting screens

alone, coupled with the measured coagulation factors, then this study would have shown a high number of false positive ICU samples for abnormal coagulation. Thus, even thought these samples showed abnormal results for coagulation factor and routine tests, global assay analysis demonstrated a normal clot formation profile after the clots were initiated and this did not tie in with the routine coagulation test results. In other words, the clotting profile, despite a prolonged initiation in clot formation was similar to that observed in healthy volunteer sample group. The haemophiliac sample group showed a more compromised clot profile overall and unlike, the ICU sample group, the clot profile in the haemophiliac sample group could be explained by their aetiology of the disorder.

First, the data from whole blood analysis by thromboelastometry, which doubled as an indirect measure of thrombin generation, showed that there wasn't a distinct difference between ICU and healthy volunteer samples. In addition, this study showed that within a cellular system, the response to rFVIIa in the ICU sample group was comparable to that observed in the healthy volunteer group. Even though both sample groups were responsive to the single rFVIIa dose, there was no distinct difference in the ROTEM parameters between the two groups. The use of rFVIIa as a haemostatic tool has been well established in controlling bleeding in haemophiliacs with inhibitors and other coagulopathies (Hedner and Ingerslev, 1998). Previous studies have shown that low-TF activated thromboelastometry is a suitable method for monitoring response to rFVIIa (Ingerslev, 2000). For this study, rFVIIa demonstrated the capability to promote haemostasis in both whole blood and plasma samples from ICU patients. The graphical display of measurable parameters allowed for analysis of the differences within the ICU sample group despite the fact that overall the ICU results were not all that

distinguishable from those observed in the healthy volunteer sample group. Monitoring the effects of rFVIIa by recording continuous whole blood coagulation was a major advantage compared with the monitoring initiation of plasma clotting. This may be because whole blood analysis was within a cellular and plasma protein system and this was a much better representation of what may be going on in *vivo* compared to analysing clotting in plasma. The most noticeable effect of rFVIIa was reflected in the CT and the CFT of coagulation, thus the initiation of coagulation. This study showed that variation in response to rFVIIa within the ICU sample group were variable might be influenced by several factors which require further analysis. However, in this study, the ROTEM assay was a good method characterising phenotypic variance amongst ICU patients as well as detecting the response to rFVIIa in an *in vitro* setting.

Plasma analysis using the CLoFAL assay proved useful in such a way that the first stage of the assay, the clot formation stage was corroborative to what the data demonstrated in the ROTEM assay. During CLoFAL analysis, the ICU samples showed a prolonged time to the clot iniitation, as observed with the CT and CFT of the ROTEM assay and the lag timeof the CAT assay. However once the clot initiated, it formed with a stability the was comparable to that observed in healthy volunteers samples. This may be attributed to the fibrin structure of the clot that was formed in these plasma samples. The structure of the fibrin formed may be affected by both he pro- and anti-coagulant pathways in these samples, therefore any alteration occurring tin these pathways will affect the structure of the fibrin clot formed and subsequently its rate of lysis.

Both the ROTEM and CLoFAL assays showed that even though the time taken to clot formation was prolonged, once activated, the clot structure was normal, or even

enhanced in the ICU samples, making both these global assays useful in depicting a more comprehensive picture of coagulation in ICU patient samples. The CLoFAL assay was able to analyse the stability of the fibrin clots formed in plasma samples in the presence of a lysing agent, tpa. The prolonged clotting screens in ICU samples may be suggestive of an inability to initiate and sustain a stable clot. In Section 3.4, it was demonstrated that fibrin clots in ICU plasma samples were not susceptibility to lysis and that they demonstrate a stabilised fibrin clot compared to the healthy volunteer group. The rate at which the fibrin clots lyse affects the stability of the clot formed, which may be attributed to the nature of the clot structure. The clot structure depends on the presence and rate of thrombin production. The data collected from this study is consistent with several factors that suggest a predisposition to abnormal thrombin production in the ICU samples. This was further supported by prolonged clotting screens and the low coagulation factor levels.

Areas of future analysis

At present, one of the known common factors in patients on the ICU is that they have varied pathology and variable underlying and predisposing factors all which contribute to their clinical state and several of these factors can exist simultaneously. Even though I was able to show, in this study, that the initiation stage of clot formation is prolonged in the ICU samples, and that the subsequent stages of coagulation is comparable to healthy volunteers sample coagulation or enhanced, this response was not unified within ICU group. The response within this group was varied and there are various factors about ICU coagulation that this study did not address due to several limitations one of which was sample quantity.

One of the key areas of further study in the ICU sample group that links coagulation and fibrinolysis is the thrombi activatable fibrinolysis inhibitor (TAFI) pathway. In the presence of high thrombin levels, TAFI is activated, which then suppresses plasminogen activation of plamsin and therefore prevents the degradation of the clot to soluble fibrinopeptides. Previous reports have suggested that TAFI deficiency plays a significant role in the ICU setting (Meijers *et al*, 2000). Its deficiency has been linked to predisposing to a bleeding diathesis because of an impaired capacity of the coagulation system to protect clots from lysis (Watanabe *et al*, 2001; Meijers *et al*, 2000; Van Theil *et al*, 2001) although it has been reported that a decrease TAFI level does not increase the risk of plasma lysis (Lisman *et al*, 2001). Studies have also shown that the ability of rFVIIa to stabilise coagulation in haemophiliacs with inhibitors is TAFI dependent (Lisman et al, 2002). Investigating the role of TAFI may play a pivotal role in elaborating on the coagulation in critically-ill patients.

Another area for further investigation is the fibrin clot formation structure in ICU samples. A previous study was able to measure fibrin formation, turbidimetrically, by determining mass/strength ratio of the fibrin fibres formed in the clot. By measuring the turbidity at a single wavelength, clot structure was determined under multiple conditions (Wolberg, 2007). This study was able to demonstrate the nature and type of fibrin fibres formed within the clot will determine its susceptibility to plasma lysis. The same approach can be applied to the ICU sample group.

Low-TF activated global assays in this study have demonstrated that samples from ICU patients have delayed activation coagulation but once initiated can be normal or enhanced. This further clarifies current reports on how routine coagulation tests are not accurate predictors of haemostasis outcome in patients with complex coagulation.

However, several investigations need to be carried out to link relevant end points of either bleeding or thrombosis to these global assays with ICU patients so that a broader understanding of their haemostasis is achieved.

CHAPTER 5:

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APPDENDIX

Assay Development
Appendix i

- **Assay development of an *in vitro* assay that utilises a single tissue factor concentration [TF] for optimal clot formation**

The TF was added to operate as a physiological activator. The main reason for this was to ensure that the TF concentration added to the plasma was sufficient to trigger clot formation. This test aimed to locate the lowest threshold TF concentration to initiate clotting in these samples.

Titration of various innovin TF concentrations were analysed using pooled platelet poor plasma and ROTEM assay parameters measured. Previously, there have been variations in TF concentrations that are standardised among investigation groups for processing and assay conditions (Sorensen *et al*, 2003 (**a**) and Sorensen *et al*, 2004 (**b**)). Therefore, in light of this, a single exogenous optimal TF concentration was standardised to activate clot formation on samples analysed on the ROTEM.

TF titration was performed using multiple dilutions (Table i) of the TF stock solution (9.3nM) ranging from 1:5 dilution (~1.86pM) to 1:20,000 dilution (~0.465pm) as starting concentrations. In each step, 20µl of the respective TF concentration was added to 300µl of pooled platelet poor plasma (PPP) obtained from healthy volunteers and the reaction initiated with 20µl of $Cacl_{2.}$

Dilution	~ Starting concentration, ρM	~ final TF concentration, ρM in 340µl of plasma
1:5	1860	109.4
1:10	930	54.7
1:25	372	21.9
1:50	186	10.9
1:100	93	5.5
1:500	18.6	1.1
1:1000	9.3	0.5
1:2000	4.65	0.27
1:5000	1.86	0.11
1:10,000	0.93	0.05
1:20,000	0.465	0.03

Table i TF titrations with corresponding concentrations in pM

Method: Plasma samples were run using the ROTEM assay and the respective TF added and the clotting times recorded. This was preformed in triplicate.

Results:

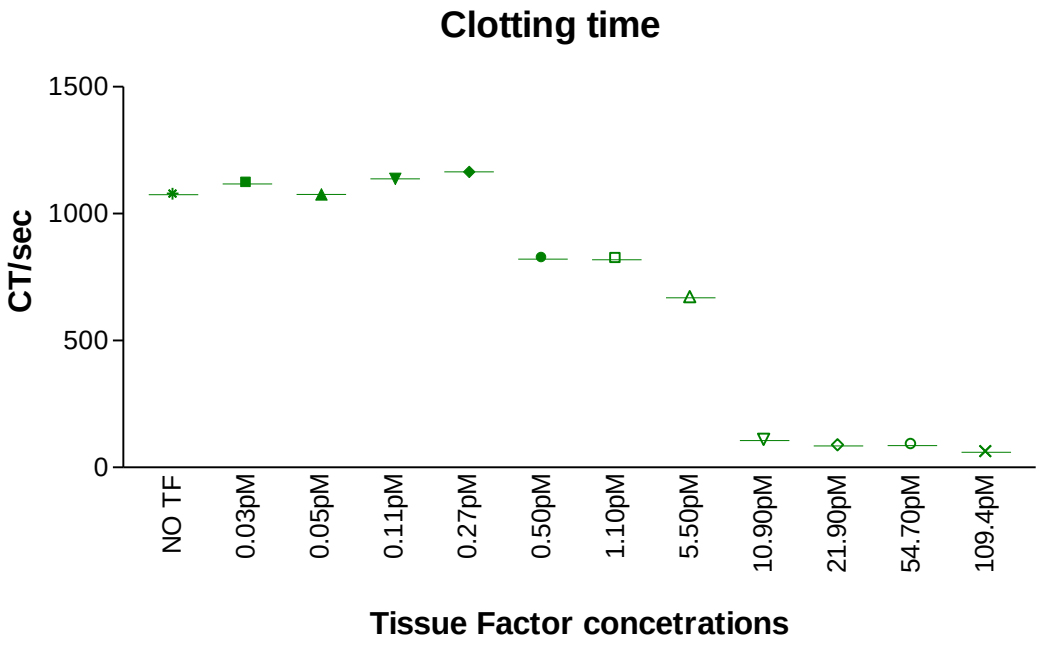

Figure i Tissue factor concentrations and the times taken to clot formation in normal platelet poor pooled plasma, results are expressed as the mean of triplicates and the bar represents the mean of the triplicates

The results of the clotting time in Figure i showed that there is no significant change in the CT up to 0.27ρM. CT decreased at the TF concentration of 0.50ρM when the clotting time was reduced in the plasma sample. After this point, clotting times follow a decline and reach a plateau phase at a concentration of 10.90ρM that continues for the reminder of the TF concentrations. Statistically, there was no significant difference between clotting times of endogenous TF in the sample and concentrations from 0.03ρM - 0.27ρM. However, the variation in TF concentration noted between 0.50ρM – 109.4ρM showed a statistical significance in clotting times ($p<0.01$ *ANOVA*). The results indicate that the lowest TF found to induce clot formation was at a concentration

of 0.50ρM. The concentration of TF used to trigger clot formation in this plasma assay was 0.5ρM.

Appendix ii

- **Assay development of an *in vitro* assay that utilises a single volume of rFVIIa for the purpose of analysing fibrin clot responses to rFVIIa**

In order to investigate the response of the analysis sample to rFVIIa, a volume titration of 8.82ng/ml was carried out to ascertain a standard volume of rFVIIa to add to the sample during global assay analysis. The changes in plasma clotting profile on the ROTEM assay were analysed the in the presence of rFVIIa.

Method: ROTEM assays were conducted with the infusion of rFVIIa at a concentration of 0.6mg/ml (standard therapeutic concentration per 90kg weight) at variable volumes and fibrin clot formation recorded. rFVIIa volumes were tested on normal pooled plasma and results recorded as an average of triplicates.

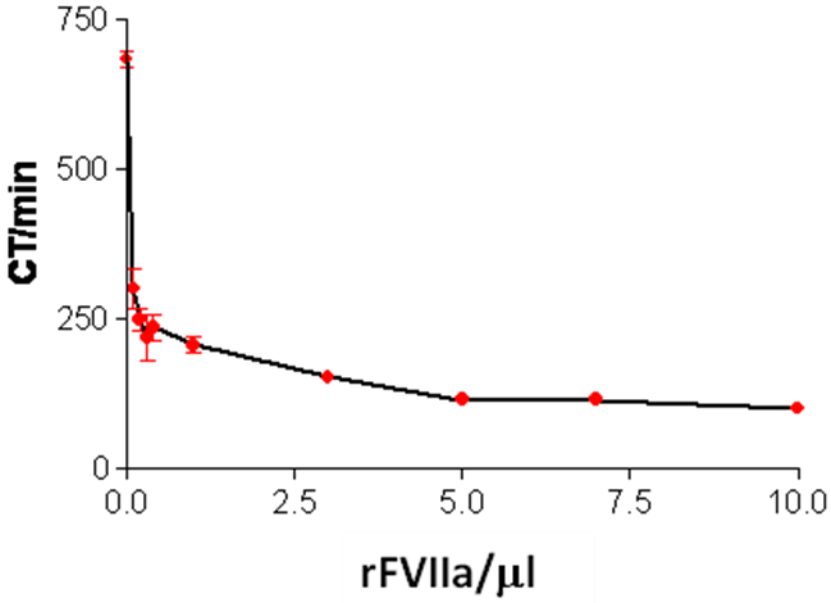

Figure ii rFVIIa volume titration in normal platelet poor pooled plasma, results are expressed as the mean of triplicates and the bar represents the mean of the triplicates

Results: Clotting time was proportional to the volume of rFVIIa added to the plasma samples up to a volume of 5.0µl (*p<0.0001*).. The maximum amplitude and clot angles (*not shown*) did not show any significant trends that correlate with the titration volumes and reach a plateau at much lower volumes (0.5µl), which may the influence of rFVIIa in a plasma system (see section 3.4). This study showed that the optimal volume of rFVIIa required to be used for the ROTEM assay that uses a sample volume of 300µl is 5µl.

Appendix iii

- **Assay development of an *in vitro* assay that utilises a single tpa concentration for optimal clot lysis**

The tpa used was added to operate as a physiological activator of clot lysis. This test aimed to locate the optimal threshold tpa concentration to initiate lysis in these samples. This study, designed to optimise a tpa concentration, was carried out by titration of 2-chain tpa concentrations and their influence on clot lysis using the CLoFAL assay on normal platelet poor plasma was analysed. The tpa titrations was were diluted from a tpa stock solution (1mg/ml) ranging from starting concentrations 20µg/ml to 500ng/ml dilutions.

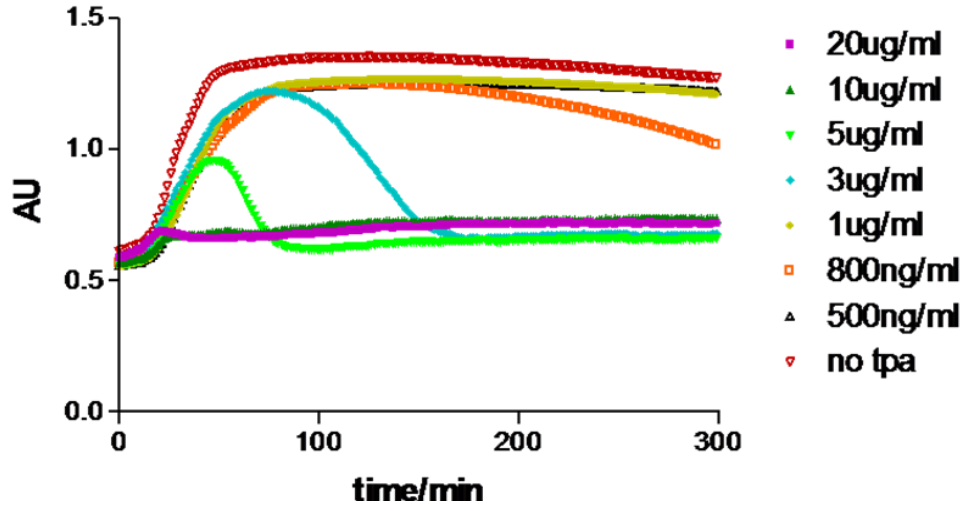

Figure iii tpa titration in normal platelet poor pooled plasma, results are expressed as the mean of triplicates and the bar represents the mean of the triplicates. AU = Absorbance units

The results shown in Figure iii demonstrate that the increase in concentration of tpa induced fibrin clot lysis. 3µg/ml of tpa allowed for clot formation and induce clot lysis. Concentrations of tpa higher than 3µg/ml induced clot lysis too early or the toa

concetrations lower than 3µg/ml did not induce lysis. For the CLoFAL assay the concetrtion of tpa that was employed to indice lysis was 3µg/ml.

Appendix iv

- **Assay development of an *in vitro* assay that utilises a single PL concentration for optimal clot lysis**

The CLoFAL assay involved the addition of 25µl of phospholipid (PL) to the plasma or reaction lysis buffer mixture, respectively. Therefore a titration of PL concentrations was performed to determine a concentration for the assay.

The results in Figure iv show the plasma turbidity is inversely proportional to the PL concentration. The concentration of PL that gave the highest signal of absorbance was at 100µM. 100µM concentration of PL was used in the CLoFAL assay, giving a final concentration of 12.5µM.

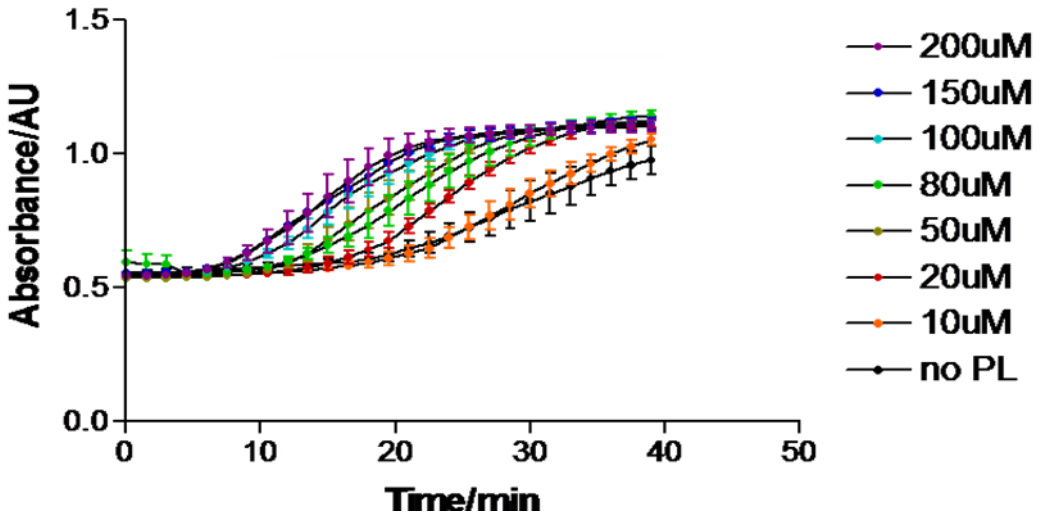

Figure iv PL titration in normal platelet poor pooled plasma, results are expressed as the mean of triplicates and the bar represents the mean of the triplicates AU = Absorbance units

Appendix V

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DE MONTFORT UNIVERSITY
LEICESTER • BEDFORD

01 February 2006

Brenda Sempasa
Dept Of Haematology
Addenbrookes NHS Trust
Po Box 234
Cambridge
CB2 2SQ

Dear Ms Sempasa

Re: Ethical Approval Application for '*Hamestatic effects of activated recombinant factor seven (rFVIIa).*'

I am writing regarding your application for ethical approval for a research project titled to the above project. This project has been reviewed in accordance with the Operational Procedures for De Montfort University Faculty of Health and Life Sciences Research Ethics Committee. These procedures are available from the Faculty Research and Commercial Office upon your request.

I am pleased to inform you that ethical approval has been granted by Chair's Action for your application '*Hamestatic effects of activated recombinant factor seven (rFVIIa)'*. This will be reported at the next Faculty Research Committee, which is being held on Thursday April 13th 2006.

Should there be any amendments to the research methods or persons involved with this project you must notify the Chair of the Faculty Research Ethics Committee immediately in writing. Serious or adverse events related to the conduct of the study need to be reported immediately to your Supervisor and the Chair of this Committee. Also, The Faculty Research Ethics Committee should be notified by e-mail to HLSFRO@dmu.ac.uk when your research project has been completed.

Yours sincerely,

Professor Paul Whiting
Chair
Faculty of Health and Life Sciences
Research Ethics Committee

Professor G Grant
Dean

Faculty of Health and Life Sciences, The Gateway, Leicester LE1 9BH.
Tel: (0116) 255 1551 / **Fax:** (0116) 257 7135 / **Email:** GGrant@dmu.ac.uk

Appendix VI

CAMBRIDGE LOCAL RESEARCH ETHICS COMMITTEE NHS

Box 148
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Hills Road
Cambridge CB2 2QQ

Chairman: Dr G E Berrios
Administrator: Elaina M Friend
Telephone: 01223 217983 Internal: (151) 3983
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Dr Trevor P Baglin,
Box No 234
Dept. of Haematology
Addenbrooke's NHS Trust
Hills Road
Cambridge
CB2 2QQ

13 August 2003

Dear Dr Baglin,

REC Ref: 03/041 **Improving diagnostic accuracy in patients with symptomatic bleeding disorders by use of global tests of blood clotting**

Thank you for your letter dated 26th June 2003 clarifying the points raised by the Local Research Ethics Committee.

As agreed by the Committee at its meeting on Friday 21st February 2003, I am taking Chairman's Action to approve this project. This approval is for three years from the date of this letter.

A list of the documents that have been reviewed and approved by the Committee is attached.

Conditions of approval:

- That the Patient Information Sheet and Consent form be labeled as follows: Version 2 dated 26th June 2003
- The protocol agreed must be followed and any changes will require prior LREC approval.
- Any serious or unexpected adverse events must be reported to the LREC, study sponsor and other local investigators.
- A progress report should be completed one year from the date of this letter and every 12 months thereafter whilst the study is ongoing. A final report must also be sent to the LREC within 3 months of the research being completed.
- The LREC has given approval for the study on ethical grounds only, therefore it is still necessary for you to obtain approval from the R&D Director or relevant management of the host organisation in which the work will be done.

Furthermore, whilst I am sure that every effort is already made to preserve the confidentiality of any patient information used in this study, could you please ensure that the team of investigators and everyone who has access to patient information appreciates the importance of maintaining that confidentiality, particularly in respect of the use of computers and the statutory regulations laid down in the Data Protection Act 1998.

The Cambridge Local Research Ethics Committee operates in accordance with ICH Good Clinical Practice Guidelines.

Yours sincerely

Dr GE Berrios MA (Oxon) MD FRCPsych FBPsS FMedSci
Chairman
Local Research Ethics Committee